EFFECT OF PHOTOPERIOD AND DIETARY STRATEGIES ON CROP MICROBIAL ECOLOGY AND HEALTH OF BROILER CHICKENS

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ABSTRACT

Increased restriction of antibiotic use in the poultry industry has focused research efforts towards alternative strategies to promote bird health. Photoperiod and nutritional management strategies are recognized as major factors affecting bird health and were investigated here as strategies to modulate immune status and intestinal microbial abundance. In the first study four experiments, each comprising more than 5,000 broiler chickens, were conducted to examine the effect of photoperiod duration on immune parameters and livability assessed by mortality incidence. All birds were exposed to 23L:1D (23L) photoperiod from 0-7 d of age prior to photoperiodic treatment allocation. In the first two experiments, broiler chickens (Ross 308 and 708) were housed under 14L:10D (14L), 17L:7D (17L), 20L:4D (20L) and 23L:1D (23L) and raised on used litter. Photoperiod treatments included 15L:3.5D:2L:3.5D (15L), 17L, 23L in experiment 3 and 13L:11D (13L), 18L:6D (18L), 23L in experiment 4. In experiment 3 and 4, Ross 308 were fed different dietary amino acid levels (Low-Lo, Medium-Med and High-Hi). Several immune parameters (relative organ weight, heterophil:lymphocyte (H:L) ratio, response to systemic lipopolysaccharide, systemic vaccination antibody response, and a whole blood chemiluminescence (WBCL) assay) were used to monitor immune status of birds across these experiments. Near continuous photoperiod (23L) reduced liver and bursa relative weight concomitantly with increased incidence of total and infectious mortality. However, there was no consistent evidence of compromised innate or acquired immunity nor of altered response to systemic inflammatory challenge.

In a second study, the effects of photoperiod (13L, 18L and 23L) and dietary amino acid level (low, medium, high) on performance, breast meat yield and crop microbial ecology were measured in 39 d-old broiler chickens. Increased photoperiod duration of ≥18L reduced the relative crop size and increased crop pH compared to 13L. Increased photoperiod (23L) and the highest amino levels increased final body weight and breast meat yield. Short photoperiod (13L) increased feed efficiency and microbial abundance in the crop, mainly of *Lactobacillus* spp. as evaluated here by T-RFLP profiling and qPCR enumeration techniques.

The final experiment was a growth trial where broiler chickens were fed three diets with three primary ingredients (corn vs wheat vs pea), ground using a 350 mm (fine) or 1000 mm (coarse) hammer mill screen size, and offered *ad-libitum* or in three meals per day. Final body weight of broiler chickens was not affected by dietary ingredients or grind size except meal-fed broilers had the lowest body weight compared to *ad-libitum* fed counterparts. 16S rRNA gene based T-RFLP profile analysis of crop contents and crop mucosa associated community revealed broilers fed corn- or wheat- based diets segregate from broilers fed pea diets, however, no clustering of ileal content T-RFLP profiles was observed. Quantitative PCR analysis of crop content further revealed higher abundance of *Lactobacillus* spp., *L. johnsonii* and *L. salivarius* in broilers fed corn and pea-based diets, while in crop mucosa pea diets promoted increased abundance of *L. gallinarium* versus corn and wheat-fed birds. Three times per day meal feeding increased ileal counts of *L. johnsonii*, *L. gallinarium* and *L. reutri* compared to *ad-libitum* fed broiler birds. This study demonstrated that easily digestible substrates (e.g. available starch, amino acids) might serve as an important substrate for the proximal broiler chicken intestinal microbial community, particularly *Lactobacillus* species which further can be stimulated by diet composition, processing and feeding strategies.

Overall, findings here suggest the importance of photoperiod management and dietary composition on bird health, emphasizing a possible important role of crop microbial composition.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACTH	adrenocorticotropic hormone
ad-lib	*ad-libitum*
AGE	agarose gel electrophoresis
AGPs	antibiotic growth promoters
AgRP	agouti-related protein
AME	apparent metabolisable energy
AMPK	adenosine monophosphate-activated protein kinase
ANS	autonomic nervous system
AOAC	Association of Official Analytical Chemists
AVOVA	analysis of variance
BBS	bombesin
bp	base pair
BSA	bovine serum albumin
Bwt	body weight
CCK	cholycestokinin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CNS	central nervous system
Co	coarse
Con A	concanavalin A
cpm	counts per minute
cpn-60	chaperonin-60
ct	threshold cycle
D	dark
d	day
DGGE	denaturing gradient gel electrophoresis

DNA	deoxyribonucleic nucleic acid
dNTP	deoxyribonucleic triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EEG	electroencephalogram
ELISA	enzyme linked immuno sorbent assay
ERRs	extra-retinal photoreceptors
EU	European Union
F	female
FCM-FISH	flow cytometry-fluorescent *in-situ* hybridization
Fi	fine
g	gram
GIT	gastro-intestinal tract
GLM	General Linear Model
GnRH	gonadotropin releasing hormone
GRF	growth hormone releasing factor
h	hour
H:L	heterophil : lymphocyte
HBSS	hank's balanced salt solution
Hi	high
HPA	hypothalamic–pituitary–adrenocortical
i.v.	intravenous
Ig	immunoglobulin
IL	interleukin
INL	intermittent photoperiod
Kg	kilogram
L	duration of light exposure per day
L. acidophilus	*Lactobacilli* acidophilus
LPS	lipopolysaccharide

Lo	low
M	male
Med	medium
mf	meal fed
mTOR	mammalian target of rapamycin
NDV	New Castle Disease virus
NPY	neuropeptide Y
NRC	National Research Council
NSPs	nonstarch polysaccharides
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PHA	phytohemagglutinin
POMC	proopiomelanocortin
PRL-r	prolactin receptor
PWM	pokeweed mitogen
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RDP	ribosomal database project
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
s	seconds
SCFAs	short chain fatty acids
SEM	standard error of the mean
spp.	species
SRBC	sheep red blood cells
TAE	tris-acetate-EDTA
Temp	annealing temperature
TRF	terminal restriction fragment

T-RFLP	terminal-restriction fragment length polymorphism
UPGMA	unweighed, pair group method using arithmetic averages
UV	ultraviolet
vs	versus
WBCL	whole blood chemiluminescence
WBCs	white blood cells
Y=mx+b	Y=dependant variable; x=independent variable; m=slope; b=intercept

1.0 LITERATURE REVIEW

1.1 Photoperiod Detection and Response in Poultry

In a natural setting, photoperiod or duration of light exposure per day (L), provides relatively accurate cues to poultry about the immediate and anticipated environmental conditions, which are important in determining the appropriate physiological responses. Darkness stimulates melatonin secretion from the pineal gland and calibrates the internal, biological clock of the bird. The presence or absence of melatonin controls daily and seasonal rhythms of various physiological systems (Pang et al., 1996; Hriscu, 2004) of host. The physiological systems influenced include the cardiopulmonary, reproductive, excretory, thermoregulatory, behavioral, immune, and neuroendocrine systems. This light-pineal-melatonin mechanism is essential for the initiation of physiological acclimatization and for animal's survival in a changing environment (Dawson et al., 2001; Goldman, 2001).

Annual changes in photoperiod length have been exclusively associated with the seasonal regulation of reproduction, molting and song events in wild birds such as sparrows (house, white-crowned and tree), stonechats, finches and European starlings (Dawson et al., 2001). Generally, in the majority of wild birds, long photoperiods initiate gonadal maturation after an increase in secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus. After the breeding phase, a photo-refractory stage follows, causing regression of the gonads simultaneously with a reduction in GnRH concentration, thus initiating molting (a seasonal replacement of the plumage). Exposure to short photoperiods is crucial for the termination of this photorefractory phase in a seasonal breeder (Turek, 1975; Dawson et al., 2001). Exposure, once again, to long photoperiods initiates gonadal maturation and the cycle repeats.

Reproduction and molting (both energy consuming and mutually exclusive processes) in wild birds, typically occur at a time of year when food resources are plentiful, and with the molt phase usually following the reproduction or breeding period (Dawson et al., 2001). Non-photoperiodic cues, like food availability and internal circannual rhythms, provide fine-tuning to the photoperiod control of the seasonal breeding processes of wild birds (Wingfield, 1980).

In addition to the pineal gland's role in light detection, the photoreceptors in avian eyes are also important for the reception of photoperiod cues. The cues received in the retinas primarily influence the bird's behavior, activity and performance. Birds can additionally perceive a fourth type of light wavelength, i.e, ultraviolet (tetra-chromatic color vision) in comparison to humans, who can primarily detect three wavelengths; red, green and blue (tri-chromatic vision) by single-cone photoreceptors (Wineland, 2002). Independent of the eyes and pineal gland, direct photoreception in birds is mediated through extra-retinal photoreceptors (ERRs) located in the hypothalamus, which primarily influences reproduction (Benoit, 1964; Turek, 1975; Lewis and Morris, 2000; Dawson et al., 2001; Wineland, 2002). Benoit (1964) discovered quite early that photoperiod-induced reproductive organ growth could be stimulated in domestic ducks that were blinded (covering the eyes, surgical removal of the eyes or the excision of the optic nerve). Similarly, Foster and Follet (1985) proposed the involvement of a rhodopsin-like-photopigment that was independent of the eyes, as being responsible for the uninterrupted reproductive response in Japanese quail. Furthermore, these ERRs have also been identified in fish, reptiles and amphibians, fascinatingly demonstrating that even blind animals of these species can respond to light, carry out photoperiod-induced reproductive changes and entrain circadian rhythms (Binkley, 1993).

The effects of photoperiod in commercial poultry have been reviewed by Manser (1996), Olanrewaju et al. (2006) and Schwean-Lardner et al. (2016). These reviews highlight the marked importance of photoperiod in controlling both behavioral and physiological processes affecting bird reproduction, health and performance in broiler chickens. Various studies have identified photoperiod as one of the most potent environmental factors affecting reproduction (Etches, 1998), performance (Buyse et al., 1996; Abbas et al., 2008; Schwean-

Lardner et al., 2012 a, 2016), behavior (Schwean-Lardner et al., 2012b), welfare (Gordon, 1994; Buyse et al., 1996; Manser, 1996; Özkan et al., 2006, Schwean-Lardner et al., 2016), immune status (Abbas et al., 2008), incidence of mortality (Schwean-Lardner et al., 2013), diurnal feeding patterns (Savory, 1976a,b; Schwean-Lardner et al., 2014) and health (Schwean-Lardner et al., 2013) of poultry raised in environmentally controlled housing units. Across the world, various types of photoperiod regimes are practiced in the poultry industry. These programs typically adjust light duration and intensity over a 24 h period (sometimes longer) with the aim to optimize performance and profitability. Savory and Duncan (1982) illustrated in domestic fowl that, undoubtedly, these birds have tremendous capability of adapting to a wide array of photoperiods. However, if given the option, hens preferably stay under illuminated areas and tend to spend at least 80 per cent of their time (> 19 h) in light (Savory and Duncan, 1982). Extended or continuous photoperiod programs are commonly implemented with an aim to maximize feed intake and growth of broiler chickens (Onbaşılar et al., 2007; Schwean-Lardner et al., 2012a, 2013, 2016). However, recent studies (Schwean-Lardner et al., 2012a,b, 2016) with broiler chicken have shown superior growth can be achieved by 17L:7D (17L) and 20L:4D (20L) in comparison to 23L:1D (23L) and 14L:10D (14L) at 32 days (d) of age. On other hand, Coban et al. (2014) showed that Ross 308 male broilers had an increased stress level as indicated by higher heterophil:lymphocyte (H:L) ratio when housed under continuous photoperiod (24L:0D), in comparison to birds under a 16L:8D or a self-determined photoperiod (24 L and free choice of Darkness).

There is, however, a mounting body of evidence available (Abbas et al., 2008; Schwean-Lardner et al., 2013; Zheng et al., 2013; Coban et al., 2014), indicating improved overall bird health (ocular health, welfare, livability, performance and immune status) with use of photoperiod regimes with increased darkness. The Codes of Practice for Canadian broiler production has recently been approved suggesting a minimum of 4 h of continuous dark period exposure (http://www.nfacc.ca/codes-of-practice/chickens-turkeys-and-breeders) is required per day (d) to improve bird welfare and health. However, European Union Council Directives 2007/43/EC (Lewis et al., 2009) and Schwean-Lardner et al. (2012a,b; 2013; 2016) both

suggested a minimum of 6 h of darkness is required to support ocular health, welfare, livability and performance in broiler chickens kept under artificial photoperiod.

1.2 Photoperiod Effect on Immune Status of Poultry

Bird's immune system relies immensely on photoperiod duration (Panshikar and Haldar, 2009), in general short photoperiod exposure has been suggested to increase immune respose in comparsion to long extended photoperiod schedules (Olanrewaju et al. 2006; Abbas et al., 2008; Zheng et al., 2013). Kliger et al. (2000) found broilers under an intermittent photoperiod (1L:3D) have improved immune responses, documented by higher proportions of splenic T-lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$) in comparison to constant (23L:1D) or intermediate (12L:12D) photoperiod raised littermates. Similarly, in dairy cattle, a short photoperiod (8L:16D) exposure has demonstrated a positive effect on the immune system and performance of animals, primarily due to prolactin action on various tissues receptors (Dahl and Petitclerc, 2003). Dry cows (Dahl and Petitclerc, 2003) exposed to a short photoperiod (8L:16D) versus long photoperiod (16L:8D) exposed animals demonstrated an increase in prolactin receptor (PRL-r - a cytokine receptor on lymphocyte) gene expression, lymphocyte proliferation and chemotaxis response.

The avian immune system provides disease resistance and protection against infection which are critical for survival and production. The immune response of any species can be broadly divided into innate and acquired categories, based on the time, stage and type of cells involved (Figure 1.1). The innate immune system is the non-specific defense mechanism of the host. It makes up the first line of defense while the acquired immune system generates a highly specific targeted response against a particular antigen (Grogan et al., 2008).

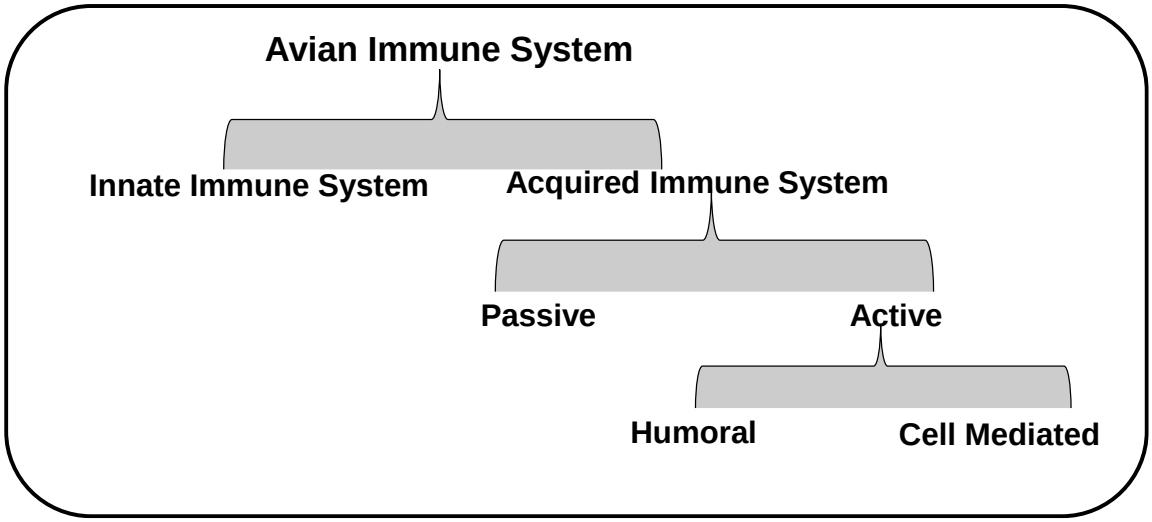


Figure 1.1. Brief overview of avian immune system.

1.2.1 Innate Immune Response

The innate response lacks specificity and targets all invading organisms. In poultry (Grogan et al., 2008) it constitutes the first line of defense to the entry of foreign pathogens and includes physical (skin and mucosa) and chemical (gastric secretions) barriers, blood proteins (complement) and phagocytic cells (heterophils, macrophages, natural killer cells and thrombocytes). Several immune parameters in poultry like heterophil:lymphocyte ratio (Abbas et al., 2008), phagocytosis index (Hriscu, 2004), organ weights (Onbaşılar et al., 2007) have been commonly evaluated to assess the innate immune system under different photoperiod regimes.

In poultry, heterophils are the counterpart to the mammalian polymorphonuclear neutrophil, serving as a cellular component of the innate immune system with its high phagocytic capacity and killing abilities (Maxwell and Robertson, 1998; Rodriguez et al., 1999). Heterophil:lymphocyte ratio is one of the prime, leading and extensively studied, innate immune status indicators of poultry housed under varying photoperiod lengths. It is usually assessed by differential counting of leucocytes (heterophils, eosinophils, basophils, lymphocytes and monocytes) on a blood smear and calculating the heterophil to lymphocyte ratio (Coban et al., 2014; Gross and Siegel, 1983). Coban et al. (2014) reported that broilers

housed under 16L:8D or given choice for darkness exposure (self-photoperiod-24 h light and free choice of darkness) have lower H:L ratios than those given no darkness exposure (24L:0D). On contrary, significantly higher H:L ratios in the non-intermittent restricted (12L:12D) group were reported by Abbas et al. (2008), when compared to birds under the intermittent (2L:2D) and continuous (23L:1D) programs. However, (Onbaşılar et al., 2007) reported similar H:L ratios for broilers housed under either continuous (24L:0D/23L:1D) or intermittent (1L:3D) programs. Lien et al. (2007) recorded no effect on H:L ratio when birds were housed under either 23L:1D or 18L:6D. In agreement, Wang et al. (2008) found no difference in H:L ratio under 23L:1D compared to an increasing photoperiod (0-3d=24L, 4-9d=10L:14D,10-16d=12L:12D, 17-22d=14:10D, 23-29d=18L:6D, 30-38d=23L:1D) programs.

Monitoring blood lecocyte oxidative burst activity is also utilized as a tool to assess poultry innate immunity status (Kogut et al., 2001; Hriscu, 2004). In general, heterophil phagocytosis elicits two processes (Kogut et al., 2001) involved in pathogen killing, one is toxic oxygen metabolite production, known as oxidative burst, and the second includes release of lytic enzymes and antimicrobial peptides from intracellular granules, a process known as degranulation. Several experiments in freshwater teleost (*Channa punctatus*), rodents and poultry have demonstrated that immune cell (heterophils and monocytes) activity and phagocytosis capacity alter with photoperiod length (Rodriguez et al., 1999; Yellon et al., 1999; Hriscu, 2004; Abbas et al., 2008; Roy et al., 2008). Assessment of the neutrophil phagocytic index (defined as the number of bacteria engulfed by 100 neutrophils) against an *Escherichia coli (E. coli)* suspension in male mice raised under a 12L:12D ± 0.5 h program revealed an apparent peak in the dark hours and a trough during light hours (Hriscu, 2004). In agreement to Hriscu's (2004) work with mice, Rodriguez et al. (1999) recorded a heightened heterophil phagocytic index (phagocytosis of inert latex beads) in ring doves (*Streptopelia risoria*) during the night period (21:30 h ± 30 min to 07:30 h ± 30 min), when housed under 14L:10D. Moreover, in these birds, the maximum phagocytic index (PI) was observed around 04.00 h and the minimum PI was observed at 18.00 h (Rodriguez et al., 1999). In Lingnan Yellow broilers, monocyte phagocytosis index, assessed as an indicator of innate immunity, increase under an intermediate (16L:2D:1L:2D:1L:2D or 17L:3D:1L:3D) versus 24L:0D (Zheng et al., 2013)

photoperiod. In contrast, housing of Siberian hamsters under a short (8L:16D) photoperiod revealed reduced phagocytosis and oxidative burst activity by both granulocytes and monocytes (Yellon et al., 1999), versus their counterparts housed under a longer photoperiod (16L:8D).

Relative organ weight is considered a relatively crude indicator of the body's capability to support the supply of different lymphoid cells (T and B cells) required during an immune response (Heckert et al., 2002; Onbaşılar et al., 2007). Exposure to darkness has been associated with increased organ weights (specifically the liver, spleen, bursa and thymus) and generally assessed as relative weight to body weight (Heckert et al., 2002; Onbaşılar et al., 2007). Significantly heavier relative organ (bursa, spleen and thymus) weights under intermittent (INL) photoperiod regimes allowing more darkness exposure (16L:2D:1L:2D:1L:2D (INL 1) and 17L:3D:1L:3D (INL II)) were recorded by Zheng et al. (2013), compared to constant photoperiod (24L:0D) exposed birds. Moreover, broilers reared under INL II, having 3 h of continuous darkness exposure, revealed a higher thymus and bursa index (4.08±0.18 g/kg bwt and 1.69±0.06 g/kg bwt, respectively) versus their counterparts under 24L:0D (thymus index of 3.19±0.12 g/kg bwt and 1.50±0.05g/kg bwt of bursa index).

1.2.2 Acquired Immune Response

The acquired immune response in any host governs specific recognition and initiation of a cascade of events directed to eliminate an infiltrating foreign pathogen or antigen (Grogan et al., 2008). Moreover, this acquired immune response also includes recalled protection during subsequent infections by any organism based on "memory cells". Acquired immunity can be categorized on the basis of the generated antibodies; active (after infection and vaccination) or passive (received preformed from mother). Grogan et al. (2008) found that this active immunity can further be differentiated primarily on basis of the type of lymphocytes involved: humoral (B lymphocytes) or cell mediated (T lymphocytes) immunity.

The assessment of the generation of the humoral and cellular immune responses in birds has been utilized by various researchers to evaluate their acquired immune-competence status (Moore and Siopes, 2000; Heckert et al., 2002; Abbas et al., 2008). Most of the research done in the field of assessing acquired immunity suggested that exposure to darkness is beneficial for

the development of the poultry acquired immune responses. Adult Japanese quail exposed to photoperiod treatments with dark periods (8L:16D and 16L:8D) generated identical and significantly heightened cellular and humoral immune responses in comparison to the responses observed in quail under constant photoperiod (24L:0D) (Moore and Siopes, 2000). In this experiment phytohaemagglutinin (PHA-P) injection was used to induce a cutaneous basophil hypersensitivity reaction for cellular immune response assessment while for the humoral immune response, primary antibody titres after intravenous injection of a Chukar red blood cell suspension, were evaluated 7 d post-injection (Moore and Siopes, 2000). In male broilers, both Onbaşılar et al. (2007) and Gharib et al. (2008) reported a negative effect on antibody production against sheep red blood cells (SRBC) and Newcastle Disease Virus (NDV) respectively, in birds housed under near constant (23L:1D) or constant (24L:0D) photoperiod, compared to birds given intermittent lighting (1L:3D). Likewise, an intermittent (2L:2D) photoperiod regimen exposure in broiler chicken revealed (Abbas et al., 2008) a significant activation of both cellular and humoral immune responses, assessed by peripheral T and B lymphocyte proliferation and antibody production, compared to the other two photoperiod regimens (23L:1D and 12L:12D).

It is still a dilemma among researchers whether the number of increased activated immune cells equips the host with better immunity against invading pathogens, or represents an activated immunity resulting from an immune-compromised state. For instance, Davis et al. (2008) reviewed various studies in birds where higher H:L ratio were correlated with better health, improved immuno-competence and improved resistance to parasites. However, in other studies low H:L ratios were correlated with less stress and better bird health. Moreover, Davis et al. (2008) highlighted studies where it was hard to decipher between stress or infection on the basis of a single or a few immune indicators (H:L ratios, organ weights, etc.). Without a better defined infection status of the individual, it is difficult to interpret results of higher circulating leucocyte, enlarged organs or increased acquired responses, which are also indicative of ongoing stress. Therefore, in context to poultry, due to presence of non-coherent scientific studies, characteristic variations in stress indicators (H:L, organ weights) among individuals, a lack of appropriate reference values, and difficulty in identifying stress condition

(acute/chronic/infection), it has been suggested that care be taken when concluding immune-competence status on the basis of a single indicator, like H:L ratio or leucocyte profile (Davis et al., 2008).

1.2.3 Incidence of Mortality

Infectious mortality is occasionally assessed in conjunction with immune status to determine the overall health of the poultry flock. Typically, a higher incidence of animal morbidity and mortality has been associated with weakened or challenged state of the immune system (Guo et al., 2010). A compromised immune system is the most common implication of chronic stress in animal which could eventually contribute to increased morbidity and mortality in response to an infectious challenge (Aapanius, 1998). In context to humans, Prendergast (2011) attempted to illustrate the effect of photoperiod on innate immunity as being responsible for the global discrepancy of infectious disease morbidity and mortality. Similarly, earlier the annual rise in infections and mortality during the winter season in both humans and several animal species was suggested primarily due to alteration of the host immune system by photoperiod exposure (Nelson et al., 1995) and may involve the effect of short photoperiod.

In broilers, while evaluating the effect of photoperiod, Lewis et al. (2009), established a profound effect of photoperiod on total mortality when photoperiod duration was > 12 h, suggesting a 0.5 % increase in mortality for each one hour (h) addition to the daylength. Coherently, increased darkness exposure of modern broiler chickens has been associated with decreased susceptibility to non–infectious (metabolic, cardiovascular and skeletal) mortalities (Abbas et al., 2008; Lewis et al., 2009; Schwean-Lardner et al., 2013). Olanrewaju et al. (2006) hypothesized that reduced mortality observed in broiler birds after extended darkness exposure may be the result of reduced early growth, improved immune status observed by higher melatonin levels and lower physiological stress indicated by lower plasma corticosterone concentrations. There are only few reports in literature directly correlating photoperiod length and the incidence of infectious mortality in animals. In rainbow trout (*Oncorhynchus mykiss*), imposition of either short (14L:10D) or long (24L:0D) photoperiod regimes increased infectious mortality approximately to 3.5-5 times as compared with a natural lighting program of 10L:14D

(Valenzuela et al., 2012). An early study conducted with broiler breeder hens suggested that the photoperiod applied during the growing phase (week 0-20) altered mortality due to a natural outbreak of Marek's disease which occurred starting at 10 weeks of age and persisted to 40 weeks of age (Proudfoot, 1970). In this study, birds were initially exposed to 17L:7D at week zero which was reduced to 9L:15D gradually to week 20. Interestingly, when dark period was added only during the evening period, mortality was 22 %, whereas when darkness was added both in the morning and evening period mortality was 44 %. However, the underlying causes for the responses in the previous study were not defined. Recently Schwean-Lardner et al. (2013) reported that infectious mortalities were reduced after darkness exposure probably due to enhanced immune status of the broilers. Similarly, Abbas et al. (2008) investigating the effect of photoperiod on broiler chicken immune status and mortality incidence observed that continuous (23L:1D) photoperiod exposed birds had three fold higher mortalities compared to an intermittent (2L:2D). This negative effect of photoperiod on broiler chciken mortality was suggested due to compromised immune status based on reduced cell and humoral mediated responses (Abbas et al. 2008).

Interestingly, thus far most of the studies (Lewis et al., 1996; Julian, 2005; Olanrewaju et al., 2006; Abbas et al., 2008) evaluating non-infectious mortalities incidences suggest that the poultry immune system improves with reduced photoperiod and increased darkness exposure. Most importantly, the negative effect of these non-infectious mortalities on the immune system cannot be underestimated as birds suffering from non-infectious diseases could probably be more prone to secondary infections or infectious mortalities, due to direct or indirect alteration of host immune system.

1.3 Mechanisms by which Photoperiod Affects Immune Response in Poultry

1.3.1 Melatonin

It has been suggested that the melatonin molecule plays an eminent role in controlling the development of the immune response and mediates the effects of photoperiod on the immune response (Rodriguez et al., 1999; Moore and Siopes, 2000; Panshikar and Haldar, 2009). More specifically, the melatonin molecule has been proposed as a key regulator in

exerting a significant effect on both cellular and humoral immune responses (Moore and Siopes, 2000) by increasing the number of splenocytes (Kliger et al., 2000), enhancing the number and activity of Natural Killer cells, and stimulating birds heterophils (*in-vivo*) and macrophages (*in-vitro*) (Hriscu, 2004). It has also been proposed that the immune system is significantly regulated by melatonin either directly by melatonin receptors identified on the surface of several immune cells (e.g. leukocytes) (Calvo et al., 1995) or indirectly through endocrine hormones which respond to melatonin (Poon et al., 1994; Hriscu, 2004).

Melatonin, a neurohormone, is secreted by the pineal gland during darkness (Zheng et al., 2013) such that plasma levels are highest under the scotophase in vertebrates, in contrast to the photo or light phase. Rodriguez et al. (1999) suggested a direct effect of melatonin on heterophil phagocytosis function in ring doves *(Streptopelia risoria)* kept under 14L:10D. In this study the highest phagocytic index occurred during the dark period (21:30 h ± 30 min to 07:30 h ± 30 min) concomitant with peak plasma melatonin levels whereas the lowest phagocytic index occurred during the photophase corresponding with the nadir in plasma melatonin (Rodriguez et al., 1999). Similarly, in mice and rats, the greatest phagocytic activity was recorded at night, coinciding with highest melatonin concentrations (Hriscu, 2004). Compared with a constant photoperiod (24L:0D), Zheng et al. (2013) showed that broilers housed under either of the two intermittent photoperiods (16L:2D:1L:2D:1L:2D or 17L:3D:1L:3D) had higher serum melatonin concentrations at both 21 and 50 d of age. The higher melatonin concentrations coincided well with evidence of improved immune status, as confirmed by heavier organ weights, an improved phagocytosis index and antioxidant level (Zheng et al., 2013). Pinealectomy, the removal of the pineal gland which prevents melatonin responses, lowered both the cellular (phytohemagglutinin (PHA) induced cutaneous basophil hypersensitivity test) and the humoral (anti-Chukar red blood cells antibodies) immune responses in Japanese quail (*Coturnix coturnix japonica*). This drop in immune response was restored after melatonin (50 µg/mL) administration in the drinking water (Moore et al., 2002).

Experiments in which exogenous melatonin supplementation was administered either in the feed (Gharib et al., 2008), in the water (Moore and Siopes, 2000, 2002; Moore et al., 2002) or by injection (Brennan et al., 2002) have also shown immune-enhancing effects on the host.

For instance, compared to melatonin free diets, heat stressed (35 °C) 4-6 week old male broiler chickens administered 40 ppm of melatonin in their feed and housed under 23L:1D, revealed a lower incidence of mortality and a positive effect on antibody production against SRBC (Gharib et al., 2008). Moore and Siopes (2000) studied adult Japanese quail to determine the immune enhancing effect of melatonin when kept under constant light. Melatonin was administered in the drinking water at 0.0, 0.5, 5.0 and 50.0 μg/mL for 16 h/d for 14 days. This experiment revealed a peak humoral immune response against Chukar red blood suspension with a dose of 5.0 μg/mL of melatonin, however all dosages increased the cellular immune response as evaluated by cutaneous basophil hypersensitivity reaction to phytohemagglutinin (PHA). Similarly, Gharib et al. (2008) found that heat stressed birds administered 40 ppm melatonin in their feed had fewer mortalities, improved antibody production capabilities and improved feed conversion, suggesting melatonin could play a role in alleviating the harmful effects of heat stress. Moreover, melatonin has been proposed as a strong anti-oxidant molecule in ring doves (*Streptopelia risoria*) which removes stress induced oxygen radicals produced during the daytime (Rodriguez et al., 1999).

Experiments in which melatonin was injected via several methods have also demonstrated a positive effect on several immune responses. Brennan et al. (2002) revealed the highest circulating total white blood cell counts and lymphocyte proliferation in male chickens housed under 16L:8D and injected subcutaneously with 40 mg/kg bwt melatonin in comparison to 0, 5, 10 and 20 mg/kg bwt doses. Gehad et al. (2008) indicated that a photoperiod paired with a greater amount of scotoperiod, i.e. an intermittent photoperiod (INL) such as 1L: 3D, in combination with melatonin injection, can enhance the immune response and reduce the inflammation induced by lipopolysaccharide (LPS) injection. It was revealed that compared to their continuous photoperiod (23L:1D) raised counterparts, male broiler chickens housed under INL (1L:3D) and injected intravenous (i.v.) with LPS (3 mg/kg bwt) demonstrated decreased cytokine production (IL-6 and IL-1), plasma corticosterone and body temperature assessed 3 and 12 h post injection (Gehad et al., 2008). Based on these findings, Gehad et al. (2008) suggested similar beneficial effects of both darkness and melatonin in alleviating the ill effects of LPS injection on the broiler immune system.

1.3.2 Stress

Stress is a condition in animals where environmental factors evoke adaptation processes and responses. Stress is principally addressed by the autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenocortical (HPA) axis (Mormède et al., 2007). Animals perceive varied types of stressors present in the external (photoperiod, temperature, sound, threat) and internal (growth rate, disease, injury) environment. Then animals direct body resources proportionate to the amplitude of the stress and the hormonal response (Siegel and Gross, 2000). Regardless of the magnitude of stress, responses include coordinated anatomical, physiological, biochemical, immunological and behavioral adaptations (Ewbank, 1985). It has been suggested that the nervous, endocrine and immune systems of the bird interplay, primarily functioning towards coordinating the best possible, energetically conserved resolution to the stressor. Moreover, opposite to the diurnal pattern of melatonin secretion, corticosteroid concentration in the circulating blood reaches an apex during the photoperiod (Özkan et al., 2012). These adrenal glucocorticoids are mainly involved in metabolic homeostasis during conditions of stress, along with suppressive effects on the immune system (Singh et al., 2010).

Stress in poultry can be assessed by several established and common immunological indicators including blood heterophil:lymphocyte (H:L) ratio (Gross and Siegel, 1983; Onbaşılar et al., 2007; Abbas et al., 2008), lymphocyte proliferation assay (Kliger et al., 2000; Abbas et al., 2008), phagocytosis index (Rodriguez et al., 1999; Zheng et al., 2013), antibody production (Moore and Siopes, 2000; Onbaşılar et al., 2007, Abbas et al., 2008) serum corticosteroid concentration (Olanrewaju et al., 2006; Abbas et al., 2008), and relative organ weight (Siegel and Gross, 2000; Onbaşılar et al., 2007; Zheng et al., 2013). Heckert et al. (2002) has suggested that increasing stocking density (10, 15 - commercial standard and 20 birds/m^2) caused lighter organ weights (spleen and bursa) of broilers, indicative of a compromised immune system due to increased stress. Stress associated with exposure to a continuous photoperiod (24L:0D) might compromise the immune response, resulting in suppressed antibody titres against SRBC and delayed type hypersensitivity response, compared to birds reared under 12L:12D (Kirby and Froman, 1991). It was found that 6 week-old male broilers kept under a non-intermittent, restricted photoperiod (12L:12D) were more stressed based on

their higher H:L ratios and plasma corticosteroid levels in comparison to their counterparts under a continuous (23L:1D) or an intermittent (2L:2D) program (Abbas et al. 2008). Abbas et al. (2008) proposed that longer exposures to darkness (12 h) in chicks might have caused stress, which in turn resulted in elevated corticosteroid levels and H:L ratios in these birds. Male broilers injected with LPS (3 mg/kg bwt) or saline have shown better immuno-competence (increased total white blood cells (WBCs), lower plasma corticosterone and lower body temperature), which is indicative of lower endotoxin stress, in birds kept under (1L: 3D) photoperiod allowing more dark versus birds reared under a continuous photoperiod (23L: 1D) (Gehad et al., 2008). In contrast to other experiments, Onbaşılar et al. (2007) studied the effect of two photoperiods (continuous - 24L:0D and intermittent - 1L:3D) in broilers and discovered no difference between the two programs for H:L ratios and relative organ weights.

Based on above experiments, more darkness exposure promotes a better immune function (Kliger et al., 2000), improved cellular and humoral immune responses (Moore and Siopes, 2000), a lower incidence of non-infectious and infectious mortality (Schwean-Lardner et al., 2013), higher melatonin levels (Zheng et al., 2013) and reduced stress (Olanrewaju et al., 2006).

1.4 Effect of Photoperiod on Feeding Behavior and Crop Physiology of Chickens

Food intake in poultry has high economic importance, due to its association with weight gain and body composition (Denbow, 1989, 1999; Richards, 2003). Feed intake patterns in poultry might vary due to strain, physiological state, age, nutrition, feed form (mash versus pellet) and environmental factors such as photoperiod (Denbow, 1989; Savory, 1980, 1999; Richards, 2003).

Similar to the effect of seasonal variation on behavioral (Shettleworth et al., 1995) repertoires (i.e. breeding and migration), environmental cues, such as photoperiod, regulates feeding patterns in poultry birds which eat preferably continuously during the photophase (Rovee-Collier et al., 1998; Duve et al., 2011). However, Lewis et al. (2009) and Schwean-Lardner et al. (2012b) have also reported occasional night time feeding behavior in modern broilers. The amount of feed consumed during the scotoperiod was inversely proportional to the

length of the photoperiod (≥9 h-12 h) and in birds as young as 5 d old. Feeding rhythms developed due to photoperiod (natural or artificial) exposure or by imposed feeding schedules (fasting, refeeding, alternate day feeding or meal feeding (one, two, three more times per d)) have a pronounced effect on bird physiology and production (Squibb and Collier, 1979; Rovee-Collier et al., 1998; Duve et al., 2011). These effects are the result of altered endocrine and metabolic status of the birds (Buyse and Decuypere, 2003). Continuous exposure to long dark periods in combination with short photoperiods could negatively affect production primarily due to very limited feed intake during scotophase, resulting in inferior body weights of birds (Ballard and Biellier, 1975; Savory, 1980; Buyse et al., 1993; Savory, 1999).

A bimodal feed intake curve has been reported in poultry birds kept under short photoperiod (14L:10D) regime (Ballard and Biellier, 1975; Buyse et al., 1993). Ballard and Biellier (1975) showed feed intake (Figure 1.2) was lowest [0.7 g of feed/(bird, h)] in the scotophase, then, in the first hour after lights come on, high levels [7.5-8.6 g of feed/(bird, h)] of feed intake were detected which then declined for the following 2 to 3 h before reaching a plateau [5-6 g of feed intake/(bird, h)]. This plateau remained until, approximately, the 8th hour of the photo period. After the beginning of the 9th hour, feed intake increased reaching its peak over the next 2-3 h [13 g of feed/ (bird, h)]. Finally, in the last hour of light, feed intake dropped to 9-10 g of feed/(bird, h).

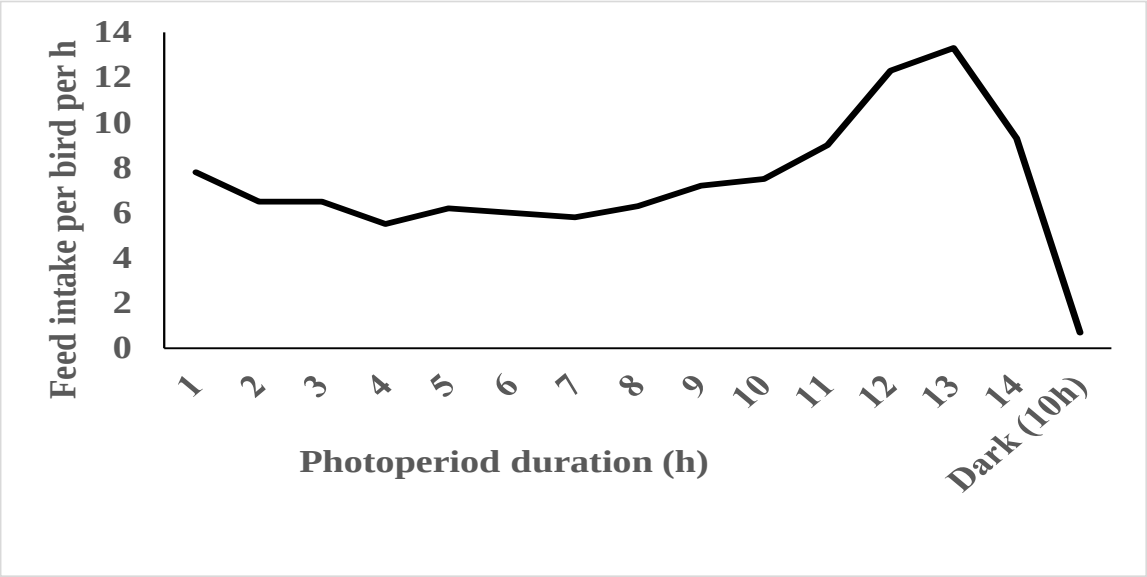


Figure 1.2. Original figure illustrating a bimodal feed intake rhythm in domestic fowl based on findings from Ballard and Biellier, 1975.

This circadian feeding pattern of increased feed intake prior to start of dark period in poultry has been attributed to cope with the nocturnal fast by storing feed in the crop (Shettleworth et al., 1995). According to Duve et al. (2011), the amount of feed stored in the crop prior to scotoperiod fulfilled approximately three-quarters of the energy needs for maintenance during the scotoperiod. In agreement, this extensive utilization of the crop for feed storage has also been reported in young turkeys (Cutler et al., 2005) and Japanese quail (*Coturnix c. Japonica*) (Boon et al., 2000) when exposed to long dark periods ≥ 10 h. In another experiment, only broilers housed under a continuous 8 h dark period exhibited a diurnal feeding pattern and showed an increase in feeding activity, crop fill activity and mass of relative crop contents 3 h prior to the initiation of the dark period, in contrast to split dark period (4+4) housed birds (Duve et al., 2011).

1.4.1 Feed Intake Regulation in Poultry

The intensive genetic selection of the modern broiler for greater growth and meat yield over several generations (Lewis et al., 2009) has led to enhanced feed intake behavior lacking

voluntary control (Richards, 2003; Ferket and Gernat, 2006). It has also been reported that, compared to laying hens, broilers express more genes associated with obesity and fat deposition, and have more glucocorticoid receptors in the hypothalamus, which together account for their greater feed intake and body fat percentage deposition (Richards et al., 2010). The mechanisms regulating feed intake in poultry are responsible for fulfilling the energy requirements for growth and various physiological states (Richards et al., 2010). Feed regulation involves multiple levels of controls which are part of a multifaceted homeostatic mechanism (Denbow, 1999). It is a complex process (Figure 1.3) predominantly controlled by the central nervous system (CNS), gastrointestinal tract (GIT) system and peripheral tissues, which communicate to each other through complex interactions via secretion and action of neurotransmitters, hormones and metabolic substrates (Richards et al., 2010).

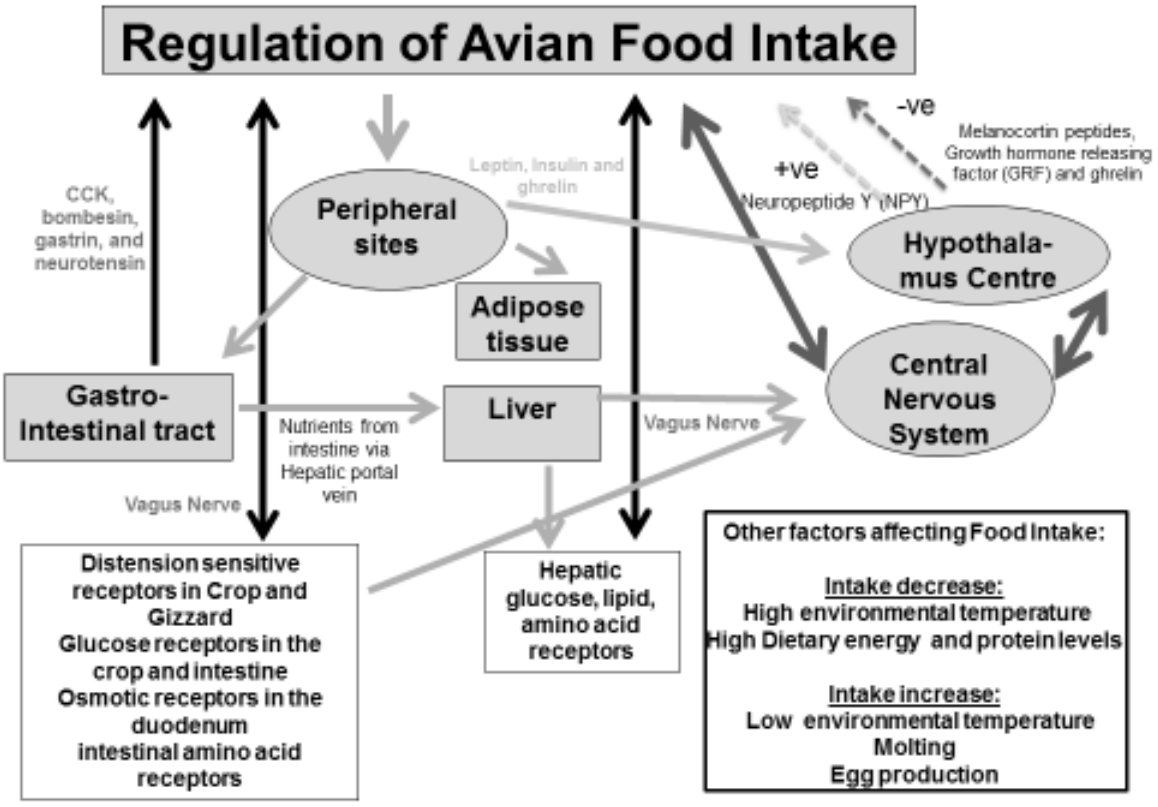


Figure 1.3. Regulation of avian food intake adapted from Richards (2003), Richards and Proszkowiec-Weglarz (2007), Richards et al. (2010) and Bungo et al. (2011).

Typically, sensing molecules identified as adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) are present in the CNS and peripheral tissues. These sensing molecules couple with unique signaling molecules (nutrients, metabolites, hormones and neuropeptides) transfer host nutritional (energy) status from the peripheral tissue to the CNS. At the central level (hypothalamus) the information gathered is interpreted and correspondingly energy intake and expenditure of the bird is modified by altering feed intake (Richards et al., 2010). For instance, CNS infusion of ghrelin, an appetite regulating peptide hormone secreted by the proventriculus, strongly prevents feed intake (Richards et al., 2010).

In the hypothalamus, two melanocortin neuron systems or feed intake regulatory circuits have been further identified: neuropeptide Y and agouti-related protein (NPY-AgRP) expressing neuron or anabolic system, while proopiomelanocortin (POMC) expressing neurons are recognized as a catabolic system. In a low energy condition, the AMPK molecule in the hypothalamus is activated. This further incites NPY-AgRP/anabolic neurons, causing an increase in feed intake and a drop in energy storage. However, mTOR stimulates POMC/catabolic neurons, resulting in reduction of feed intake, an increase in energy stores and an increase in utilization of energy for growth, maintenance, reproduction and other bodily functions (Richards et al., 2010).

Information received by the brain from visceral organs, including the gastrointestinal tract, and adipose tissue and other brain parts affects when a meal begins and ends (Forbes, 1985). The entire gastrointestinal tract, its associated organs and the liver, are involved in the peripheral regulation of feed intake in avian species. The GIT is connected to the brain via the vagus nerve. In birds, food ingestion in the upper gastrointestinal tract (crop, gizzard) often leads to the termination of feed intake due to the activation of distension osmo, chemo and thermo receptors present throughout the GIT and associated organs, and the secretion of

hormones (cholycestokinin, gastrin, bombesin) (Forbes, 1985; Husvéth, 2011). For example, feed intake ceases with the infusion of glucose, lipids, epinephrine and amino acids into the liver and additionally with the injection of peptide hormones (cholecystokinin, bombesin, and gastrin) (Denbow, 1999; Husvéth, 2011). However, differential response has been reported for broiler versus laying birds, the infusion of glucose and lipids into the liver leads to a drop in feed intake in Leghorns, but not in broilers (Whittow, 2000). Other factors such as high temperature, dietary energy and protein content usually lead to a drop in feed consumption too, whereas low temperature, energy deficient diets, molting and egg production lead to an increase in feed consumption (Husvéth, 2011).

1.4.2 Upper Gastro-Intestinal Organ (Crop and Gizzard) Role in Feed Intake Regulation

The poultry GIT organs, especially the crop, have a marginal role in feed intake regulation when food is freely available. The crop is a diverticulum of the esophagus which does not appear to play a pivotal role in digestion, but can be used as storage organ for food in case of feed deprivation (Savory, 1985; Classen et al., 2016). However, its role becomes evident during a meal or with limited access to feed, often achieved after the alteration of photoperiod and scotoperiod length (Denbow, 1999; Classen et al., 2016). The crop is not utilized during the light phase and typically remains unfilled 2.5 h before the initiation of long dark periods (Rovee-Collier et al., 1998). Birds, however, fill their crop with feed which is released slowly in the scotoperiod (Buyse et al., 1993; Duve et al., 2011) when exposed to long scotoperiod $\geq$ 8 h. In two week old male, light-bodied chicks, Barash et al. (1993) demonstrated a significantly higher relative crop and gizzard weight in meal-fed birds (1 h or 2 h /d or pair fed with 1 h group) compared to their *ad-lib* fed counterparts due to food storage in these organs. Furthermore, meal-fed (for one hour, 5 times per day) broilers reared under 18L:6D photoperiod consumed approximately two-thirds of their meal in the first 20 min, with the last third taking the remainder of the hour, leading to heavier crops (Svihus et al., 2010).

The rate of filling and emptying of the GIT, including the crop, gizzard (Richardson, 1970; Savory, 1985) and duodenum (Savory and Hodgkiss, 1984) is thought to regulate feed intake in poultry. After series of experiments in Japanese quail and domestic fowl kept under

14L:10D, Savory (1985) proposed that meal termination in birds was associated with partial crop fill and with variable amount of gizzard fill. In addition, hunger or meal initiation in most of the birds was related to fractional gizzard emptying. It appeared that half-full gizzards represent the equilibrium stage when birds are not totally hungry, nor have they attained complete satiety (Savory, 1999). At other times, the gizzard and duodenum have also been identified as candidates with better potential in affecting food intake in poultry (Savory and Hodgkiss, 1984).

Several types of sensory (chemo, osmo, thermo, distention) receptors have been identified across the entirety of the gastrointestinal tract (Gentle, 1985; Ferket and Gernat, 2006) of poultry which can impact feed intake. These receptors transmit information to the brain, where after interpretation feed intake is regulated (Gentle, 1985; Ferket and Gernat, 2006).

1.4.2.1 Stretch/Distension Receptors

A drop in feed intake has been observed after the stimulation of distension sensitive receptors in the crop of domestic fowl (Hodgkiss, 1981; Gentle, 1985). Hodgkiss (1981) has recorded electrical activity in vagal afferent fibres subsequent to the distension of the crop. Further distension of the domestic chicken crop by artificial inflation of a permanently installed balloon-cannula system lowers the amount of feed eaten during a 15-min test period (Richardson, 1970). These stretch receptors have been located in all animals (Forbes, 1985) and two different types of these receptors, rapid and slow adapting, have been identified in domestic poultry by Hodgkiss (1981). The information from these crop distension receptors is typically sent to the brain via the vagus nerve (Denbow, 1994; Svihus et al., 2010). Furthermore, information for the initiation and termination of feed intake is brought back from the CNS by a unique, avian intestinal nerve (Savory, 1999). These distension receptors have also been recognized in gizzard (Gentle, 1985). Moreover, distension of the gizzard affects feed intake more prominently than distension of the crop (Husvéth, 2011). The gizzard is therefore proposed as an additional check point in feed intake regulation due to its limited size and its ability to control the release of stored food material to the lower gut (Savory, 1999; Svihus et al., 2010). Distension sensitive receptors of the poultry duodenum have been more recognized for their ability to detect osmotic change (Ferket and Gernat, 2006).

1.4.2.2 Thermo, Osmo, Chemo-Receptors and Hormones

The presence of thermo or temperature receptors in the crop were demonstrated by cold water infusion, which was more effective in electroencephalogram (EEG) arousal than air, warm water (40 °C) or saline solution (Gentle and Richardson, 1972). These cold temperature receptors have also been identified in the poultry GIT including beak and oral cavity (Ferket and Gernat, 2006). However, the role of thermoreceptors in feed intake has not been well established in poultry (Gentle, 1985). It has been suggested that these cold temperature receptors in combination with mechanoreceptors and chemoreceptors might affect poutlry feed consumption after (chemical, textural and temperature) evaluation of ingested feed (Ferket and Gernat, 2006).

Shurlock and Forbes (1981a,b) showed that osmoreceptors (sensing osmolality changes) play a role in regulation of feed intake in domestic chicken. In cannulated adult cockerels Shurlock and Forbes (1981a) observed a significant drop in food intake for a 3 h period after the infusion of slurry diet (10 g mixed in 20 mL of water). Moreover, they recorded an equal response to highly absorbable glucose (increasing concentration and hypertonic) and non-absorbable (sorbitol and KCl) solutions infused directly into the crop. Similarly, work in unfasted, female Brown Leghorns revealed a drop in feed intake after the infusion of a hypertonic glucose solution into the crop over a 15 min test period, in comparison to 22 h fasted birds (Richardson, 1970), primarily due to osmoreceptors. Similar to the crop, a significant drop in feed intake was observed within an hour of infusions of hyperosmotic solutions of sorbitol and KCl (3 osm) into the duodenum (Shurlock and Forbes, 1981b; Forbes, 1985; Gentle, 1985). Infusion of hypertonic potassium chloride solution was shown to decrease food intake via detection by osmotic receptors in the crop and duodenum (Ferket and Gernat, 2006).

Savory (1999) later suggested that the administration of nutritive or non-nutritive agents directly into the crop of birds causes a drop in feed intake due to the stimulation of the osmo

and chemo-receptors (predicting chemical fluctuations) present in this organ. It might be possible that stimulation of these receptors can directly lead to an alteration of GIT motility and secretion of gut peptides, both of which cause meal termination (Savory, 1999).

Over a dozen hormones are produced in the GIT and its associated organs. These hormones have also been identified as having a role in the regulation of feed intake (Chaudhri et al., 2006). In avian species, intravenous injection of GIT hormones bombesin (BBS), from proventriculus and gizzard, and cholecystokinin (CCK), from antrum to ileum, causes suppression of feed intake (Denbow, 1994; Savory, 1999). This effect was speculated to result mainly due to abdominal discomfort caused by abnormal intestinal responses such as gizzard contraction and intestine motility as measured through video recording, instead of a change in GI motility and increased heart rate (Denbow, 1994; Savory, 1999).

1.5 Feeding Behavior Effect on Crop Physiology (Size, pH and Microbial Ecology)

The crop epithelium is non-glandular and thus its pH is primarily determined by microbial activity and particularly lactic acid bacteria, the predominant group present in this location (Bowen and Waldroup, 1969; Hinton et al., 2000a). Typically, pH of the crop in broilers ranges from approximately 4-5 (Durant et al., 1999). However, Bowen and Waldroup (1969) recorded a constant pH of 5.1 in the crop from 19 to 28 d of age, whereas crop pH might range from 5.3 to 5.5 at 42 d of age as reported by Hinton et al. (2000b).

In addition to its role in the regulation of feed intake, the crop may play a significant role in the defense against enteric pathogens (Fuller and Brooker, 1974; Corrier et al., 1999a,b; Hinton et al., 2000a,b; Tannock, 2004; Hilmi et al., 2007). Changes in the microenvironment of the crop, including size, pH, the amount and type of microbial fermentation products and resident commensal bacteria populations (mainly *Lactobacillius* spp.), have the potential to affect the viability of pathogens entering the bird digestive tract (Durant et al., 1999; Hinton et al., 2000a; Byrd et al., 2001). For chickens, change in crop size depends upon the photoperiod duration to which they were exposed, thereby controlling the amount of feed material stored in this location. In male broilers kept under 14L:10D, Buyse et al. (1993) showed that during the daytime the crop contains a small amount of feed, while it may remain almost empty in laying

hens during photophase (Mongin, 1976). However, at the onset of the dark period, dried crop contents increased by 10.5 fold, along with a 2.76 fold increase in gizzard/proventriculus dried contents (Buyse et al., 1993). Cutler et al. (2005) observed in young turkeys, a drop in crop pH and an increase in fermentation products (volatile fatty acids) when birds increased their feed consumption just prior to the onset of the scotoperiod (14L:10D).

The crop has been recognized as an important anterior GIT organ which can affect host nutrient digestion and bacterial community colonization across the whole intestinal tract (Fuller and Turvey, 1971). This is the first organ encountered by any pathogenic bacteria upon entering the bird's gastro-intestinal tract, including zoonotic pathogens (*Salmonella*, *Campylobacter*) of concern in human health and transmitted through poultry products. Both feeding patterns and feed composition may affect crop physiology parameters important for carrying out its role as a pathogen barrier. *Lactobacillus* spp. can be isolated in abundance from the crop, however, other bacteria like enterococci, coliforms and yeasts have also been isolated from the crop (Gabriel et al., 2006). Gram positive, rod shaped lactobacilli (10^8 to 10^9 CFU) typically form a continuous layer over the crop's stratified squamous epithelium (Fuller and Turvey, 1971; Fuller, 2001, Janczyk et al., 2009). A significant number of these bacteria have been isolated from just after hatch to adulthood in both the lumen and the crop mucosa (Fuller, 1973). *In-vitro* experiments (Fuller, 1977) have shown that the presence of approximately 10^7 lactobacilli per g of crop contents can efficiently impede the growth and multiplication of *E.coli*. Interestingly, fasting in chicks for 16 h still results in the presence of a sizeable number of viable lactobacilli (7.5×10^5) present on the crop wall and which is adequate to impede growth of oral foreign pathogens (Fuller, 1973, 2001). Moreover, crop lactobacilli may affect their own colonization, as well as that of other bacteria, not only in crop but also down the tract, either by their physical presence or through the metabolites (short chain fatty acids - SCFAs) they produce (Fuller and Turvey, 1971).

1.5.1 Effect of Crop Microbiota on Lower Intestinal Tract Bacterial Community

Any alteration in the residential microbial community and microenvironment in the crop has the potential to affect bird health (Janczyk et al., 2009). Indigenous crop *Lactobacillus spp.*

have been suggested to play significant role in the maintainence of the crop microenvironment by fermentation of food and conserving their abundance (Fuller, 1973, 1977, 2001). Several experiments conducted by Fuller and his coworkers (Fuller and Brooker, 1974; Fuller, 1977) investigating crop microenvironment and the native bacterial population have demonstrated minor changes in levels of volatile fatty acids, a decrease in native lactobacilli count and an increase in crop pH might lead to better opportunities for the colonization of potentially detrimental organisms in the GIT. Prevention of GIT pathogen like *E. coli* colonization by commensal lactobacilli has been proposed mainly by lactic acid production which lowers pH or by attachment to intestinal sites making them unavailable to non-commensal bacteria (Fuller, 1973, 1977, 2001). For instance, optimum pH required for the growth of food-borne pathogens like *Salmonella* (6.0 to 7.5) and Enterobacteriaceae (6.0 to 8.0) is well above the normal pH of 4.5-5 found in the active crop (Durant et al., 1999; Hinton et al., 2000a).

Feed withdrawal is commonly practiced for molting of laying hens (Durant et al., 1999) and to prevent broiler carcass contamination and condemnation (Hinton et al., 2000a). However, feed withdrawal has been associated with low lactobacilli count in the crop and with an increased abundance of *Salmonella enteritidis* colonization and its migration to other organs (spleen and liver) (Ricke, 2003). Durant et al. (1999) studied the effect of forced molt (9 d feed withdrawal) of 50 weeks-old Leghorn hens on crop physiology, along with an oral challenge of *Salmonella enteritidis* organisms (10^5) administered on day 4 of feed withdrawal. Forced molting caused an increase in crop pH, which ranged from 6.1-6.25, compared to 4.96-5.33 in control birds (Durant et al., 1999). Additionally, in forced molt birds, the lactobacilli numbers in the crop contents were lower (6.28-6.82 $\log_{10}$ CFU/mL) when compared to the control birds (8.33-9.01 $\log_{10}$ CFU/mL). Lactate concentrations in the crop of control birds was also higher (24.4 -51.4 µmol/mL) than that in forced molt birds (9.1-14.6 µmol/mL). Spleen and liver, *Salmonella* invasions significantly increased from 8.3 % in control birds, to 50 % in forced molt birds. Moreover, the *S. enteritidis* caecal colonization increased; it was detected in 16.7 % of non-molted birds and in 90 % of the molted birds. In the same study, *Salmonella* was isolated from the crops of approximately 50 % of forced molt hens, whilst less than 10 % of control hens were found to be positive (Durant et al., 1999). All these changes, including lower volatile fatty

acid concentrations (total, acetic, propionic and butyric) in crop, may have stimulated the expression of the virulence gene of *S. enteritidis* controlled by the transcriptional activator HilA, necessary for its survival, colonization, growth, intestinal invasion and transfer to the liver and spleen in forced molt birds (Durant et al., 1999). In their next experiment Durant et al. (2000) further examined the effect of pH, carbohydrate (0.2 % glucose, fructose or mannose) source, 0.2 % Casaminoacids and lactate (0, 25 and 50 mmol/L) on *S. enteritidis* hilA virulence gene expression. Addition of lactate and carbohydrate sources decreased hilA gene expression whereas Casaminoacids had a minor effect. In regard to lactate supplementation, suppressive effect was more pronounced at a low pH of 4 in comparison to pH 5 and 6. Hence, it was suggested that crop lumen environmental and nutritional conditions could alter virulence gene expression of common poultry pathogens like *S. enteritidis.*

Likewise, addition in the drinking water of lactic acid (Byrd et al., 2001) or a carbohydrate cocktail (Hinton et al., 2000b) during feed withdrawal of broiler chickens decreased the counts of food-borne pathogens (*Salmonella* and *Campylobacter)* in the crop. Similar to the effects of lactate in the crop, Heres et al. (2003) reported that providing fermented liquid feed may similarly prevent *Salmonella* colonization in another anterior poultry GIT organ, the gizzard, and might retard passage of the pathogen to lower segments.

Therefore, based on above findings, feed withdrawal or absence of feed in crop has been shown to elevate the occurrence of foodborne pathogens (*Salmonella* and *Campylobacter*) in the crop, emphasizing the role of crop as important control point for preventing carcass contamination in broiler processing (Byrd et al., 1998; Corrier et al., 1999a,b; Ricke, 2003).

1.6 Poultry Intestinal Tract Microbial Ecology

For several decades, the GIT of farm animals, including poultry, has been a main area of research due to its profound effect on animal nutrition, health and food safety (Apajalahti et al., 1998). The GIT is responsible for fulfilling host body energy requirements at each and every stage of development including post- hatch periods (Yegani and Korver, 2008) and undoubtedly, the GIT's resident microbiota play a pivotal role in the growth and development of poultry (Bjerrum et al., 2006; Torok et al., 2007).

In poultry, the diverse and complex intestinal microbiota consists of bacteria, fungi, protozoa, archaea and viruses (Gabriel et al., 2006; Yegani and Korver, 2008; Saxena et al., 2016). Bacteria are the principal GIT inhabitants, outnumbering total body cells by 10 fold (Engberg et al., 2000; Gabriel et al., 2006; Stanley et al., 2014). Due to their enormous impact on host metabolism, immunity and digestive physiology, the GIT's commensal bacterial community has been extensively investigated (Gabriel et al., 2006; Yegani and Korver, 2008; Stanley et al., 2014). *Lactobacillus* has been identified as the most abundant bacterial genus attached to the intestinal epithelium along the length of the chicken GIT (Collado and Sanz, 2007; Gong et al., 2007). Especially for crop squamous epithelium, it has been proposed that receptors are present for lactobacilli only. These lactobaccili help prevent the colonization of pathogens through secretion of SCFAs and lowering pH (Fuller, 2001). As SCFAs promote GIT epithelium proliferation and differentiation potentially improving barrier function (Malmuthuge et al., 2012) whereas low pH is not favourable for most pathogens growth (Durrant et al., 1999; Hinton et al., 2000a).

Diet composition and feeding management (feeding pattern/feed withdrawal) are among major determinants of the composition of the GIT microbial communities (Koutsos and Arias, 2006; Yegani and Korver, 2008; Pourabedin and Zhao, 2015). Age, stocking density (Pourabedin and Zhao, 2015), management (Choct, 2009), infectious agents (Yegani and Korver, 2008), stressors like transportation (Line et al., 1997) and molting (Holt and Porter, 1992) are also among several other critical factors affecting the GIT resident microbiota profile in poultry. All of these factors alter the colonization balance of harmful and beneficial organisms in the GIT, which in turn has profound effects on overall host health (Stanley et al., 2014; Pourabedin and Zhao, 2015; Wilkinson et al., 2016).

1.6.1 Health and Disease

It has been well established now that the GIT microbiota community and its member species play a significant role in host health (Choct, 2009; Chambers and Gong, 2011), not only in humans, but also for livestock animals, including poultry. 'Gut health' or 'a healthy gut' typically represents a state of GIT possessing several beneficial aspects, like effective digestion

of food and absorption of nutrients, absence of GIT illness, normal and stable intestinal microbiota, effective immune status and an overall state of host well-being (Guamer, 2007; Choct, 2009; Stanley et al., 2014). In context of poultry, a healthy gut is essential to avail maximum growth accompanied by high feed efficiency (Stanley et al., 2016), whereas an unhealthy state affects the intestinal tract's ability to digest food and absorb nutrients to support growth and development, which eventually compromises performance, intestinal health and quality of products (Gabriel et al., 2006; Gong et al., 2008; Yegani and Korver, 2008).

Typically, GIT resident autochthonous microbiota members form a complex symbiotic inter-relationship with host and are thought to serve a number of functions, including aiding in the digestion of dietary components, the synthesis of essential nutrients, the fermentation of products, the salvaging of energy, trophic effects on intestinal epithelia, the development of immunity and the competitive exclusion of pathogens (Apajalahti et al., 1998; Koutsos and Arias, 2006; Yegani and Korver, 2008). In return for these benefits, bacteria receive growth supporting nutrients and an environment conducive to survival. Disturbance in this host-microbiota balance due to a variety of reasons such as infection, stress, diet, etc. can lead to detrimental effects on the gastrointestinal function and host performance and development (Guamer, 2007).

In comparison to proximal, undigested dietary ingredients (resistant starch, dietary fibre) in distal gut may have beneficial effects on broiler chicken health by promotion of growth of commensal communities, generation of fermentation products, reduction of pH and prevention of pathogen colonization (Bjerrum et al., 2006; Gabriel et al., 2006; Teirlynck et al., 2009b, Czerwiński et al., 2010; Stanley et al., 2014). Studies have indicated that proximal intestine bacteria primarily *Lactobacillus* species can efficiently utilize several readily available substrates such as carbohydrates, amino acids, peptides, fatty acid esters to support their growth and with both beneficial or adverse influence on host performance and pathogen colonization (Ricke, 2003; Langlands et al., 2004; Tannock, 2004; Dahiya et al., 2005; Gabriel et al., 2006; de Lange and Wijtten, 2010; Scanes and Pierzchala-Koziec, 2014; Pan and Yu, 2014; Stanley et al., 2014). Besides beneficial effects (Tannock, 2004) on host like inhibition of pathogen

colonization and growth, providing bacterial inoculum for lower gut, habitat conditioning, small intestine inhabitance of *Lactobacillus* species has also been associated with some host ill-effects like lipid digestion impairment, allergy and inflammatory diseases which may suppress broiler growth. Broiler chicken indigenous intestinal microbial community members can also be opportunistic pathogens. *Campylobacter jejuni* and *Salmonella enterica* are common zoonotic pathogens spread mainly by chicken (Stanley et al., 2014) and constitute part of chicken normal microbiota. In incapacitated hosts these bacteria can cross the intestinal mucosal barrier to cause systemic disorders. The inhabiting cost of these micro-organisms is typically at the expense of host animal's performance and economic losses due to carcass contamination and condemnations (Richards et al., 2005; Yegani and Korver, 2008).

1.6.2 Acquisition and Succession of Poultry Intestinal Tract Microbial Community

In recent reviews, its has been indicated that to elucidate the interaction between poultry and its resident microbiota, early 20th century research focused to identify and classify intestinal bacteria mainly by implementation of culture-based methods which currently has evolved to DNA-based, culture-independent methods (Stanley et al., 2014; Pourabedin and Zhao, 2015). Only 10 % of bacteria were estimated to be identified using culture-based approaches (Apajalahti et al., 2004). However, recent advances in molecular-based technologies have enabled investigators to characterize gut communities in much greater detail in comparison to culture-based methods. By implementation of molecular techniques, 640 different species and 140 different genera have identified so far in the chicken gut, of which more than half represent previously unknown bacterial genera (Apajalahti et al., 2004).

Typically, after hatch, the bird starts acquiring microbes from its surroundings (environment, handlers, transportation and mother) which gradually develop into a highly complex microbial community in an adult bird (Pourabedin and Zhao, 2015). An ever changing and evolving balance exists between the gastrointestinal microbiota, host physiology and diet that directly affects the initial acquisition and developmental succession, eventually resulting into an established ecosystem. It has been well established that poultry bacterial communities

alter with age in all gastrointestinal segments (Stanley et al., 2014; Pourabedin and Zhao, 2015). Moreover, the diversity of the avian bacterial community becomes more complex as one moves down the GIT (Stanley et al., 2014; Pourabedin and Zhao, 2015; Wilkinson et al., 2016).

At the time of hatch, the bird GIT was considered to be essentially sterile by most investigators (Yegani and Korver, 2008), however, this perception has recently been challenged by Wielen et al. (2002) and Pedroso et al. (2005). It was proposed that even in 1 d old chicks, a complex community of bacteria exists which chicks may have acquired from surroundings before or after the hatch period (Wielen et al., 2002; Stanley et al., 2014; Pourabedin and Zhao, 2015). Within 24 h of hatch, bacteria have been detected in the crop and their numbers increased by 10 fold on the third day in the small and distal intestine, before staying relatively constant to 30 d of age (Apajalahti et al., 2004). This is in accordance with reports of Knarreborg et al. (2002), Wielen et al. (2002) and Hume et al. (2003). Molecular based detection methods (denaturing gradient gel electrophoresis -DGGE) have revealed the presence of several bands (representative of either single microbial group members or species) in the crop of 1 d old Cobb broiler chicks (Wielen et al., 2002), ceca of 2 d old developing leghorn chicks (Hume et al., 2003) and ileum of 7 d old male Ross 208 chickens (Knarreborg et al., 2002). Lu et al. (2003) also recognized these identical age-dependent differences in the chicken ileum and caeca microbiota inhabitants, illustrating successions of microbiota with age and transformation from transient communities to one of a highly complex and evolved ecosystem. Based on 16S ribosomal ribonucleic acid (rRNA) gene sequences analysis, it was found that initially at 3 d of age, both ileum and ceaca harbor similar microbiota. Furthermore, during 7 to 14 d of age, the caecal bacterial community was identified as being a subset of the ileal microbiota, which later had eventually developed into a more complex, diversified and larger community (Lu et al., 2003).

Normally, a relatively stable, fully mature (complex and diverse) bacterial community is established at approximately 40 d of age. Additionally, it has been proposed that upper gastrointestinal tract microbiota communities stabilize within two weeks, while the distal intestine microbiota stabilize later, when the bird is 3-4 weeks of age (Rehman et al., 2007).

Until the age where an established GIT microbial community is reached, each compartment has its own unique succession of bacteria populations. Initially, in young chicks, all GIT segments harbor almost similar major members of the bacterial community (Rehman et al., 2007; Stanley et al., 2014; Pourabedin and Zhao, 2015). As the bird gets older, each segment begins to support its own distinctive bacterial community, with highest similarity of segments found in the adjoining areas (Rehman et al., 2007; Stanley et al., 2014; Pourabedin and Zhao, 2015). Wielen et al. (2002) has suggested these site specific bacterial communities could be observed after 4 d of age. Before 4 d of age, the upper GIT segments, along with the ileum, have a similar 16s rRNA gene based DGGE banding pattern. Additionally, Hume et al. (2003) proposed that in Leghorns, adjoining intestinal segments will also have nearly identical banding patterns as determined by 16S rDNA based on DGGE analysis. Similar to chickens, gastrointestinal community DNA sequence analysis in Japanese quail (*Coturnix japonica)* have revealed different bacterial community members investigated from mouth to caecum, large intestine and feces (Wilkinson et al., 2016). This indicates that colonization of dominant micro-organisms in GIT locations can be affected by unexplored host-related factors, and that adjacent segments have the highest similarity when comparing microbial communities (Wielen et al., 2002).

Several poultry studies extensively investigating gastrointestinal communities have identified that Firmicutes, Bacteroidetes and Proteobacteria are among the largest phyla, and *Clostridium, Ruminococcus* and *Lactobacillus* are the primary inhabiting genera (Rehman et al., 2007; Stanley et al., 2014; Pourabedin and Zhao, 2015). Additionally, *Lactobacillus* spp. have been identified as the predominant residing bacteria in the proximal small intestine, along with other genera (*Enterococcus*, *E. coli*, *Eubacteriium*, *Clostridium*, *Propioniobacterium* and *Fusobacterium*). In the caecum lactobacilli, coliforms and enterococci are identified as the most abundant in poultry (Rehman et al., 2007; Stanley et al., 2014; Pourabedin and Zhao, 2015). 16S ribosomal DNA (rDNA) sequencing was implemented to characterize the prominent bacterial members in the intestinal and caecal contents of chickens at 3 different ages (4, 14, and 25 d). The presence of *Lactobacillus*, *Bifidobacterium*, *Salmonella*, *Campylobacter, E. coli* and *Clostridium* was revealed in different locations, and in different relative proportions (Amit-Romach et al., 2004). Similarly, Knarreborg et al. (2002) explored the effect of dietary fat

source (soy oil or a mixture of lard and tallow) and antibiotic inclusion (a combination of avilamycin at 10 mg/kg and salinomycin at 40 mg/kg of feed) in Ross 208 male birds ileal community at different ages (7, 14, 21, and 35 days) using culture and PCR-DGGE analysis. They found that the ileum microbiota at 7 d -35 d of age consists of several predominant bacterial targets (*Lactobacilli*, fermentative and non-fermentative Enterobacteria, *C. perfringens*) and Streptococci, Enterobacteria, and *C. perfringens* abundance increased with age of bird. Moreover, dietary inclusion of antibiotics resulted in lowered ileal *Lactobacillus* counts at 14 and 21 d old chickens whereas addition of soy oil in diets reduced ileal *C. perfringens* counts (Knarreborg et al., 2002). The use of 16S rDNA based probes in investigating the caecal community of Cobb broilers at 4, 14 and 25 d of age revealed that at 4 d of age lower *Lactobacilli* (25 %) was detected in ceca compared to *Salmonella* (40 %), along with *Campylobacter* (2 %), *Clostridium* and *E. coli* species (Amit-Romach et al., 2004). Later at 14 d *Bifidobacteria* was identified, and together *Lactobacilli* and *Bifidobacterium* accounted for approximately 40 % of the total bacteria, while *Salmonella* counts decreased and *E.coli* and Clostridium counts remained constant. At 25 d of age *Lactobacillus* and *Bifidobacterium* constituted almost 50 % of the caecal bacterial species detected. *Salmonella* counts further decreased and *E.coli* and *Clostridium* counts rose to constitute approximately 30 % of the total bacterial member species (Amit-Romach et al., 2004).

1.6.3 Poultry Upper Gastro-Intestinal Tract Segments Microbial Ecology

1.6.3.1 Crop

Microscopic examinations of crop epithelium scrapings revealed the presence of a 2-3 cell thick layer of Gram positive, rod shaped *Lactobacilli* associated with the stratified squamous epithelium (Fuller and Turvey, 1971; Fuller, 2001, Janczyk et al., 2009). Typically, their number can reach up to 10^8 to 10^9 CFU per g of crop contents (Fuller, 1973, 2001; Guan et al., 2003). In an unfed state (such as a nocturnal fast) this lactobacilli count can drop to 10^6 but still, this count is sufficient to give the host bird protection from pathogenic bacteria it would receive through the feed (Fuller, 1973, 2001). In addition to lactobacilli, other bacterial like enterococci, coliforms and yeasts along with several other like *E. coli*, *E. fergunsonii*, *Klebsiella*

ozaenae, K. pneumoniae, Staphylococcus lentus, Micrococcus luteus, Enterobacter aerogenes, Pseudomonas aeruginosa, Eubacterium spp., and *Sarcina* spp., have also been isolated from the crop (Gabriel et al., 2006; Rehman et al., 2007).

In agreement with previous culture-based methods, recent molecular-based studies in the chicken have shown lactobacilli as the most predominant bacterial group inhabiting the crop (both in lumen and attached to the mucosa) (Hilmi et al., 2007; Gong et al., 2007; Rehman et al., 2007; Janczyk et al., 2009). The crop microbiota changes significantly within the first week of a broiler's life, while a stable community isn't established until after 14 d of age (Guan et al., 2003). In day old birds, the lactobacilli number was 5.84 ± 1.23 $\log_{10}$ CFU/g, and which increased 1,000-fold within one week, reaching 9.00 ± 0.41 $\log_{10}$ CFU/g (Guan et al., 2003). Wielen et al. (2002) reported similar findings with less than five 16S rRNA gene-based DGGE bands, representative of bacteria members, observed in crop of one-day old broiler birds. Later, these DGGE band numbers increased until 11 d of age, followed by a decline during the 11 d to 28 d period and after 28 d again an increase in DGGE band numbers was observed.

In recent reviews (Stanley et al., 2014; Pourabedin and Zhao, 2015) researchers compared the resident microbiota in different intestinal segments and reported that the crop was the least diverse niche among all GIT locations examined, indicating microbial community members residing at this site belong to related or identical groups of microbes mainly, lactobacilli. However, species differences between *Lactobacilli* have been reported previously. Hilmi et al. (2007) examined crops of 1 and 5 week old broilers and discovered an abundance of *Lactobacillus* species; *L. reuteri* (33 %), *L. crispatus* (18.7 %), and *L. salivarius* (13.3 %) (based on partial 16S rRNA gene sequencing). Hilmi et al. (2007) further proposed that irrespective of farm origin and feed, in young chickens *L. reuteri* was the prevalent resident. But later, in adult birds, it is not among the principal *Lactobacillus* species predominant in the crop. Earlier, Guan et al. (2003) revealed an age-related succession in the crop of broilers, of the inhabitant Lactobacillus species microbiota; *L. reuteri, L. johnsonii*, *L. crispatus*, *L. gallinarum* and *L. amylovorus* were regularly isolated at all ages, while *L. acidophilus* (identified from 7-35 d) and *L. salivarius* (identified from14-42 d) were identified later in life.

Additionally, the crop microbiota community in laying hens raised under 16L:8D and fed basal diets (wheat, corn, pea) with or without naturally occurring green algae (*Chlorella vulgaris* @5.0 g/kg of diet), was investigated by 16S rRNA gene based PCR-DGGE fingerprint analysis. There was an abundance of bands for *Lactobacillus* including *L. gasseri, L. salivarius* and *L. gallinarum,* for this GIT section (Janczyk et al., 2009).

The crop's mucosa-associated microbiota analysis in broilers (Collado and Sanz, 2007) by culture-based and flow cytometry fluorescence *in-situ* hybridization (FCM-FISH) methods, showed the presence of Atopobium, Bifidobacterium, *E. coli*, and *Eubacterium rectale–Clostridium coccoides* as being in abundance at this location. Culture-based analysis of the crop mucosa revealed that *Lactobacillus* and *Propionibacterium* were the principal residents at this location. The total anaerobic bacterial counts were 7.12 (log CFU/ cm^2 of crop tissue) and *Bacteroides, Clostridium, Enterobacteriaceae, Enterococcus, Staphylococcus* and yeast were present at 3.77, 4.21, 4.27, 3.64, 4.45 and 4.03 log CFU/ cm^2 in the crop tissue, respectively. Contrary to the distal gut section, *Bifidobacterium* counts (6.55 log CFU/cm^2 of crop tissue) were detected at significantly higher levels in the crop mucosa (Collado and Sanz, 2007). Analysis of the crop mucosa using 16S rRNA gene cloning and sequence analysis of the associated bacterial community in broilers revealed a similar dominance of *Lactobacillus* species where *L. salivarius* and *L. johnsonii* were the most abundant members detected (Gong et al., 2007). Identical to crop lumen, lactobacilli, enterobacteria, Gram-positive cocci and enterococci have also been reported adhering to crop wall (Rehman et al., 2007).

1.6.3.2 Proventriculus and Ventriculus

In poultry, after passage through the crop, ingested food passes through the proventriculus to the ventriculus or gizzard (Scanes and Pierzchala-Koziec, 2014). In poultry, the proventriculus is the "true" stomach where acidic conditions (caused by secretion of HCl to approximate pH of 2.0) start the digestion of the ingested food. In contrast, the gizzard aids in mechanical breakdown of food (Scanes and Pierzchala-Koziec, 2014). Pantin-Jackwood et al. (2005) have reported an inability to detect bacteria by culture or histological examination from the proventriculus of broilers. Additionally, there is a scarcity of literature available describing

the indigenous microbial community of these two adjoining GIT locations. It is mainly due to the highly acidic and reducing environment in these organs which leads to low numbers and heterozygosity of bacterial counts (Rehman et al., 2007; Stanley et al., 2014). However, healthy proventriculus is crucial for better performance and feed conversion along with its importance for carcasss contamination and food safety (Huff et al., 2001; Pantin-Jackwood et al., 2004).

At the next GIT location, the gizzard, some investigators have failed to identify any micro-organisms due to extreme growth conditions, low pH and faster digesta passage rate. Contrarily, others have detected a diverse population of bacteria, although in relatively low abundance dominated primarily by lactobacilli and *Clostridiaceae,* similar to the crop (Gabriel et al., 2006; Rehman et al., 2007; Stanley et al., 2014). Several culture-based investigations (Rehman et al., 2007) of broiler chicken gizzard contents (log cfu/g) has identified total anaerobes (7.0-8.1), *Lactobacillus* spp. (6.2-7.5), *E. coli*/ coliforms (3.4-4.8) and *Enterococcu*s spp. (4.3-5.1). Analysis using 16S rRNA showed that *Lactobacilli* were one of the major bacterial groups inhabiting the gizzard, whereas *L. aviaries* and *L. salivarius* were present in abundance in the mucosa (Gong et al., 2007; Rehman et al., 2007).

1.6.3.3 Small Intestine

Similar to the GIT's anterior locations, the chicken duodenum and jejunum harbor low counts of the main bacterial groups. A low abundance and counts of bacteria (10^3-10^5 cells/g digesta) at these locations is probably due to the continued presence of hostile conditions (low pH, high oxygen pressure, digesta reflux and digestive secretions such as enzymes and bile salts) and a high digesta passage rate (Gabriel et al., 2006; Rehman et al., 2007). Wielen et al. (2002) evaluated the duodenal DGGE banding pattern at different ages (1, 4, 7, 11, 15, 22, 28 and 39 d) of Cobb broiler chickens. An increase in duodenal band numbers was observed during 1 to 11 d of age which was followed by a decrease in the number of bands detected from 11d to 28 d. However, after 28d of age, duodenal DGGE band numbers again revealed an increase. Engberg et al. (2000), using culture-based methods, revealed the presence of total anaerobes, coliform, lactose-negative enterobacteria, *Lactobacilli*, other lactic acid producing bacteria and enterococci at both duodenum and jejunum (counts ranging from 4.32 to 8.97 cfu/g of contents).

Moreover, *Lactobacilli* have been found in abundance in these proximal gut sites (Rehman et al., 2007; Stanley et al., 2014) and which has been shown to increase with age (Amit-Romach et al., 2004).

The comparison of mucosa-associated microbiota (Gong et al., 2007) belonging to different chicken gut segments reveals that the duodenum has the most heterozygous community, followed by the jejunum and then the ileum. Furthermore, Gong et al. (2007) suggested *L. aviaries* and *L. salivarius* as the principal occupants in the duodenum, jejunum and ileum mucosa, whereas *Arthromitus* was detected primarily in the jejunum and ileum mucosa. Culture-based identification of mucosa-associated communities by Collado and Sanz (2007) of total anaerobes, *Lactobacillus*, *Propionibacterium*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, Enterobacteriaceae, *Staphylococcus* and yeast yielded mean values of 7.20, 6.32, 5.90, 2.82, 4.24, 3.50, 4.50, 2.30 and 1.83 log CFU/ cm^2 of duodenal tissue in broilers, respectively. Additionally, *Atopobium* and *C. histolyticum* were detected in the duodenal mucosa by FCM-FISH in same birds. In 25 d-old Cobb chickens, Amit-Romach et al. (2004) identified *E. coli* and *Clostridium* in the duodenum and ileum using 16S rDNA targeted probes.

The ileum environment, unlike that of the duodenum or jejunum, becomes more favourable for bacterial growth due to low oxygen pressure, enzyme and bile salt concentrations (Gabriel et al., 2006; Rehman et al., 2007; Stanley et al., 2014), which elevates bacterial counts to 10^9 bacteria per g of ileal contents (Apajalahti et al., 2004). Microscopic examination of ileal digesta shows that on average counts are 10^8 to 10^9 cells per g, while the ileal mucosa harbors approximately 10^{11} bacterial cells per bird (Gong et al., 2002). An age dependent evolution of the microbiota has been well studied in the ileum (Knarreborg et al., 2002; Lu et al., 2003; Gong et al., 2008; Rehman et al., 2007). Gong et al. (2008) detected an increase in DGGE bands in the ileum with an increase in the age of broilers; at 3 d there were 7-8 bands, which later increased to 30-40 bands in a 42 d old chicken. Wielen et al. (2002) based on DGGE banding pattern analysis in commercially raised broilers proposed that the ileum microbial community only remains constant during 11 to 28 d of age. However, in contrast, Lu et al. (2003) proposed that the ileum had a stable bacterial community from 7 to 21 d of age and again between 21 to

28 d of age, but that there was a very unique ileal microbial community structure from 3 and 49 d of age.

It has been suggested that of the main facultative anaerobes, *Lactobacillus* spp. were the predominant ones detected in the ileum, followed by enterococci and coliforms, as analyzed by molecular based methods (Apajalahti et al., 2004; Gabriel et al., 2006; Stanley et al., 2014). Culture-based methods used by Knarreborg et al. (2002) also confirmed lactobacilli in abundance in the ileum (> 10^7 cfu per g of ileal contents, assessed at 7, 14, 21 and 35 d of age). Partial 16S rRNA sequence analysis in chickens revealed *Lactobacillus* as the chief inhabitant (70 %), followed by *Clostridiaceae* (11 %), *Streptococcus* (6.5 %) and *Enterococcus* (6.5 %) in this niche (Lu et al., 2003). Furthermore, Olsen et al. (2008) reported 4.5 x 10^8 total bacterial cells, 5.60 x 10^6 *Clostridium*-like bacteria and 6.61 x 10^8 *Enterobacteriaceae*-like bacteria per g of ileal digesta (with the prevalence of *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Clostridiaceae* and *Bacteroides*-like bacteria) investigated by 16S rRNA targeted fluorescence in situ hybridization (FISH) technique. *Streptococcus* has also been isolated at 14 d of age and *C. perfringens* at 35 d of age (Knarreborg et al., 2002). *L. johnsonii* and *L. crispatus* were identified at all ages by DNA sequencing of ileum samples, unlike *L. salivarius*, which was detected only at 35 d of age (Knarreborg et al., 2002). Harrow et al. (2007) detected a higher number *L. salivarius* species in the ileum, followed by *L. johnsonii* by using real time qPCR. A divergent bacterial community profile (mainly Gram positive with low G+C contents) in the lumen and mucosa of the ileum from six week old broilers was identified using terminal-restriction fragment length polymorphism (T-RFLP) and 16S rRNA clones sequence analysis (Gong et al., 2002). Based on partial sequencing analysis of 16S rRNA, it was proposed that *Lactobacilli* and *Enterococcus cecorum* are the predominant bacterial groups found attached to the ileal mucosa. This analysis demonstrated that out of 51 cloned sequences nearly half (23) were related to *Lactobacilli*. Out of the *Lactobacilli* relatives, *L. aviaries* were the most abundant species in the broilers fed corn-soy diets. T-RFLP analysis after Msp1 revealed three distinct bands (90, 405 and 415 bp) from the content and mucosa samples of the ileum. The 90 bp band was present only in the mucosa, whereas the other two bands (405 and 415 bp) were detected in the digesta samples (Gong et al., 2002). Similarly, 16S rRNA gene based PCR-

DGGE band profile analysis in broiler ileum mucosa-associated bacterial community revealed three mucosa specific bands, which differed from those found in the luminal contents (Malmuthuge et al., 2012). However, the total bacterial population enumerated by 16S rRNA based qPCR revealed no differences between the ileal mucosa and lumen bacterial communities (Malmuthuge et al., 2012).

1.7 Conclusions

Management of photoperiod is one of the most commonly implemented strategies across the world to avail maximum production in commercial poultry system including broilers. Short photoperiods paired with longer dark periods (6-8 h) has been indicated to positively affect the development of both the innate and acquired immune responses in broiler chickens. Simultaneously, short photoperiod has also shown to promote diurnal feeding pattern, utilization of the crop for food storage, the crop microbial community and overall gut health. Unfortunately, there is no consensus in the reviewed literature on the impact of photoperiod on the innate and acquired immune responses. Disagreements too exist regarding the relationship between crop fill and the microbial ecology in broilers. Few studies have investigated all these factors; photoperiod duration, immune status, crop microbial ecology, all together in one model. Therefore, the overall objective of the following studies was to investigate the effect of a range of photoperiod programs and nutritonal strategies, widely prevalent in the poultry industry worldwide, on the immune status and microbial ecology in the upper gastrointestinal tract of broiler chickens.

1.8. Hypothesis and Objectives

Based on the literature review above it was hypothesized that photoperiod length and variation in feeding strategies affect immune status and microbial ecology of the crop in commercial broiler chickens with important implications for bird health and performance. The primary objective of each experiment contributing to the overall objective, is outlined as below:

Chapter 1: The objective of the first study was to examine the effect of photoperiod length on the immune status and incidence of infectious mortality of broilers in four experiments. Several

innate and acquired immune parameters were assessed, along with the incidence of infectious mortality.

Chapter 2: The objective of the second experiment was to investigate the effect of photoperiod and dietary amino acids content on performance, breast meat yield and microbial ecology of the crop in broiler chickens.

Chapter 3: The objective of the third experiment was to evaluate the effect of dietary ingredient type, feeding frequency and feed form on microbial ecology of the crop and ileum in broiler chickens.

2.0 EFFECT OF PHOTOPERIOD ON IMMUNE STATUS OF BROILER CHICKENS

2.1 Abstract

Four experiments were conducted to investigate the effect of photoperiod on immune status in broilers chickens raised under simulated commercial conditions. All birds were exposed to 23L:1D (23L) photoperiod from 0-7 d of age, after which selected photoperiod treatments were imposed in experiments 1 and 2 (14L, 17L, 20L, 23L), experiment 3 (15L:3.5D:2L:3.5D, 17L, 23L) and experiment 4 (13L, 18L, 23L) such that for each experiment photoperiod was replicated in 2-3 separate rooms of at least 450 birds per room. Relative organ weight, heterophil:lymphocyte (H:L) ratio, humoral reponse to vaccination, heterophil function assay and chronic lipopolysaccharide (LPS) challenge were used to assess immunity. In experiment 1 and 2 broiler (Ross 308 and 708) birds were raised on used litter using non-medicated diets to give an early exposure to microbial challenge. Moreover, in these two experiments (1 and 2) at 19 d and 27 d of age selected male Ross 308 birds (n=64) were chronically challenged with LPS (i.v.) to assess the response to inflammatory stress. In experiment 3 and 4 humoral immunity was assessed in female Ross 308 broilers only, following *E. coli* toxoid vaccination. Additionally, in both these experiments (3 and 4) immunization and heterophil function was assessed using a whole blood chemiluminescence (WBCL) assay. Finally, total and infectious mortality incidence was also assessed as a measure of livability. Initially, post one week of photoperiodic treatment installation, exposure to 13L (experiment 4) or 14L (experiment 1) significantly reduced relative weight of spleen compared to 23L. Continous exposure of broiler chickens to 23L reduced relative liver (26 d of age) weights in experiment 4 and bursal weights (21 d of age) in experiment 1 versus regimes allowing > 7 h of darkness exposure. The H:L ratio was not affected by photoperiod in experiments 3 and 4. Moreover, the functionality of heterophils, as assessed by WBCL assay, and the serum antibody response to *E. coli*

toxoid vaccination were also unaffected by photoperiod. Nonetheless, a higher incidence of infectious and total mortality was recorded in broiler birds given 23L in experiment 4. Chronic challenge with LPS reduced body weight gain during the challenge period, although the effect was significant in the first experiment only. The relative weights of spleen and liver were increased by LPS challenge in both experiments, whereas relative weight of bursa and the H:L ratio were unaffected. Taken together there were indications that 23L photoperiod reduced liver and bursa weight in younger birds associated with increased incidence of infectious mortality. However, there was no other evidence of compromised innate or acquired immunity nor of altered response to systemic inflammatory challenge.

2.2 Introduction

In the last few decades, livestock species, particularly meat type poultry, have been extensively selected for rapid growth rate (Olanrewaju et al., 2006). Photoperiod, duration of light exposure per day (L), is one of the commonly implemented management tools used by poultry producers to maximize feed intake and support rapid growth. However, besides growth, photoperiod has the potential for altering the immune status of birds (Abbas et al., 2008, Abbas 2013, Zheng et al., 2013, Schwean-Lardner et al., 2016). Several photoperiod regime recommendations and guidelines (EU Council Directive, 2007) are available in the literature (Abbas et al., 2008; Schwean-Lardner et al., 2012a,b, 2013, 2016) or independently (National Farm Animal Care Council, 2016) recommending that at least one 4-6 h long continuous dark period to be included in the photoperiod program for better livability and immune status of broilers, without compromising the desired body weight gain.

Across the world, there is prevalence of several types of photoperiod programs (increasing, restricted, intermittent, short, long and continuous/near-continuous) which often interplay with duration of day and light intensity over a 24 h period for manipulating broiler growth depending upon market requirement. Commonly, commercial broiler chickens are raised under continuous (24L:0D) or near-continuous photoperiod (23L:1D),

under the presumption that it will increase their body weight and feed intake, resulting in a greater profit for the producer (Abbas et al., 2008, Schwean-Lardner et al., 2012a, 2016; Zheng et al., 2013). Extended photoperiod regimes are preferred in the broiler industry, but many studies have suggested that these, in combination with contemporary high performance genetic lines, may be responsible for compromised bird immune responses (Kogut, 2009; Zheng et al., 2013) and increased mortality (Gordon, 1994; Campo and Dávila 2002; Abbas et al., 2008; Schwean-Lardner et al., 2012a, 2016). This may be because exposure to an extended photoperiod with short or no dark (D) period, might act as an environmental stressor leading to immunosuppression (Kirby and Froman, 1991; Kliger et al., 2000; Zheng et al., 2013). Both acquired (Abbas, 2013) and innate (Zheng et al., 2013) responses were depressed in broilers raised under 23L/24L compared to photoperiod schedules offering more darkness exposure (16L:2D:1L:2D:1L:2D, 17L:3D:1L:3D and 2L:2D). Similarly, in adult Japanese quail (*Coturnix coturnix japonica*) exposure to long (16L:8D) or short (8L:16D) photoperiod resulted in similar and higher cellular and humoral immune responses versus continous photoperiod exposed birds (Moore and Siopes, 2000). Similarly, rearing of broiler chickens under continuous or near-continuous photoperiod was shown to adversely affect recognized innate immune responses indicators, including heterophil to lymphocyte (H:L) ratio (Gross and Siegel, 1983; Moore and Siopes, 2000; Campo et al., 2007; Coban et al., 2014) antioxidant status and organ weights (Zheng et al., 2013). In contrast, several others did not observe photoperiod effects on immune response (Blair et al., 1993; Campo and Davila, 2002; Lien et al., 2007; Onbaşılar et al., 2007). Moreover, immune parameter results are also subject to an interpretation bias; whether greater values of immune status indicators such as H:L ratios, organ weights and levels of cytokines represent better immune capabilities or an on-going stress condition of the host (Prendergast et al., 2003; Smith and Hunt, 2004; Davis et al., 2008).

A compromised immune system may consequentially influence a bird mortality and morbidity incidence. Annually huge economic losses are incurred by the poultry industry as a result of production losses due to infections mainly of bacterial, viral and protozoan

origin. Additionally, due to globalization, there is constant danger of exotic and emerging diseases (Kogut, 2009). Adding to the problem is the latest concern over the use of antibiotics in animal production. Hence, there is an urgent need of practical and safe alternatives for the prevention and control of these infectious agents. Strategies which act through immunological interventions to lessen the microbial pathogen load and which boost host immunity and the innate immune system to control and clear infections, would be of great significance for both the poultry industry and its consumers (Kogut, 2009). Manipulating the photoperiod could become one such safe alternative. The literature is contradictory regarding the effect of photoperiod on the incidence of total mortality in poultry, however, most agree that shorter photoperiods reduce mortality (Downs et al., 2006; Lien et al., 2007; Hassanzadeh et al., 2016). Until now, despite its huge importance, only a few reports have directly investigated the effect of photoperiod on infectious mortalities of commercially raised broiler chickens (Schwean-Lardner et al., 2013, 2016).

The discrepancy in the literature regarding the effect of photoperiod on the immune status and livability of broiler chickens has prompted us to investigate the effect of different photoperiod schedules on poultry immune function by assessing innate and acquired immune responses using a variety of methods across several large studies using large numbers of birds.

2.3 Materials and Methods

All experimental protocols involving animals were approved by the University of Saskatchewan Animal Care Committee and followed recommendations and guidelines of the Canadian Council on Animal Care (1993).

2.3.1 Bird Management and Housing

Four different experiments were conducted to study the effect of photoperiod exposure on the immune status in broiler chickens at the Poultry Centre facility, University of Saskatchewan. Photoperiod programs (13L:11D-13L; 14L:10D-14L; 15L:3.5D:2L:3.5D-15L; 17L:7D-17L; 18L:6D-18L; 20L:4D-20L; 23L:1D-23L) providing darkness (1-11 h) either in a single continuous or split pattern, were evaluated over the

course of four experiments (Table 2.1). In all experiments day-old birds were exposed to 23L:1D photoperiod regimen with a light intensity of 20 lux until 7 d of age, after which the selected photoperiod treatments with 10 lux light intensity were randomly allocated to the 8/9 rooms depending on experiment (Table 2.1). Each room was subdivided by using a removable penning system into 12 pens each of 2.3 x 2.0 m in dimension. During each experiment, the lights came on at 07:00 (dawn beginning at 06:00) for all photoperiod treatments, except for the 15L regimen where lights came on at 00.30. Diets were formulated according to Aviagen recommendations (2007), which either met or exceeded the nutrient requirement recommendations for broilers by National Research Council (NRC) (1994). All starter and grower diets were fed in a crumble form and finisher diets were fed in pelleted form (4.7 mm diameter). Moreover, birds had *ad-libitum* (*ad-lib*) access to corn-soy based diets and water until they were marketed at either 38/39 d or 49 d of age (experiment-dependent).

Table 2.1. Experimental details to investigate effect of different photoperiods on immune status in broiler chickens.

Experi-ment	Genotype (Ross X Ross)	Photoperiod program	Total Number of Birds	Age (d) at Marketing	Litter	Housing Density
1	308/708	14L, 17L, 20L, 23L (Continuous dark periods)	5800 (1400 M,1500 F per genotype)	39	3rd time used	30 kg/m^2
2	308/708	14L, 17L, 20L, 23L (Continuous dark periods)	5400 (1400 M,1300 F per genotype)	48	4th time used	30 kg/m^2
3	308	15L:3.5D:2L:3.5D, 17L, 23L (Split dark periods)	6500 (3000 M, 3500 F)	38	Fresh	30 kg/m^2
4	308	13L, 18L, 23L (Continuous dark periods)	6500 (3100 M,3400 F)	39	Fresh	30 kg/m^2

L= hours of light and D= hours of darkness.

2.3.2 Experiments and Data Collection

2.3.2.1 Experiments 1 and 2

The impact of four photoperiod regimes 14L:10D (14L), 17L:7D (17L), 20L:4D (20L) and 23L:1D (23L), sex and genotype on the immune status of broilers was investigated. A total of 5,800 and 5,400 broiler birds were used in experiments 1 and 2 respectively, which included both male and female birds belonging to Ross X Ross 308 or 708 genotype kept in separate pens. Hatching eggs for Ross 708 were supplied by Aviagen from the United States, while Ross 308 eggs were sourced from Lilydale breeder flocks. Eggs were hatched by Lilydale Foods in Calgary and chicks were feather sexed and then delivered to the University of Saskatchewan. Built-up wheat straw litter was used for a third time in experiment 1 and for a fourth time in experiment 2 to give broilers an increased microbial exposure early in their life. In addition, diets were without anti-coccidial drugs or growth promotants. The vaccine Coccivac®-B (Schering-Plough Canada Inc.) against *Emeria* species was administered to all day-old chicks at the hatchery before transportation to the Poultry Centre. Eight rooms were used in each of these experiments, allowing two replicates per photoperiod treatment. Out of 12, only four pens were dedicated for immune data collection, each having 70 birds per pen.

Body weight and relative organ (liver, spleen, bursa) weights, expressed as g of tissue/g of body weight, were recorded weekly (14, 21, 28, 35 d) in experiment 1 and only at the end of the trial (48 d) in experiment 2, by euthanizing five birds per pen (from a total of four dedicated pens/room), across all rooms (n=160). In both these experiments eight male birds from each room (n=64) were selected, weighed and wing banded and given a chronic inflammatory challenge by administering (i.p.) 2 mL saline containing 400 µg bacterial endotoxin lipopolysaccharide (LPS) every second day (Webel et al., 1998) over a 10 d challenge period (from day 17 to 26 in experiment 1 and from day 27 to 36 in experiment 2). Male control birds (n=64) were administered (i.p) 2 mL sterile saline. At end of the 10 d challenge period a blood sample was collected for H:L ratio evaluation by superficial venipuncture of the brachial vein from each bird before cervical dislocation and

then final body weight and relative organ (liver, spleen and bursa) weights were recorded (n=128). Briefly, a blood sample for measurement of H:L ratio was collected into 2.0 mL Vacutainer tubes (Becton, Dickinson and Company, New Jersey, USA) containing EDTA as anticoagulant. Immediately after collection, to prevent clot formation, blood and EDTA were mixed by placing tubes on a mixer and then kept on ice. Duplicate blood smears were prepared for each bird, smears were let to dry for 24 h and stained using Wright-Giesma Stain (EMD Chemicals purchased from VWR, Lot number 4215). The number of heterophils and lymphocytes were counted under oil immersion microscopy until 60 cells were counted (Gross and Siegel, 1983; Zulkifli et al., 2000).

2.3.2.2 Experiments 3 and 4

Three photoperiod regimes were examined in these two experiments to study impact of photoperiod, sex and diet on immune status of Ross x Ross 308 broilers. The 23L photoperiod regime was compared to 15L:3.5D:2L:3.5D (15L) and 17L:6D (17L) in experiment 3 and to 13L:11D (13L) and 18L:6D (18L) in experiment 4. For each feeding broiler phase, diets were formulated to low (Lo), medium (Med) or high (Hi) dietary amino acid levels. All diets were formulated on the basis of ideal protein concept, where all other essential amino acids were balanced in proportion to lysine (Aviagen Broiler Nutrition Specification, 2007). In these experiments a total of nine rooms were utilized at the facility, allowing three replicates per photoperiod treatment in each experiment. A total of 6,500 day-old broiler chicks of both sexes was used and raised separately in 6 pens in each room to assess photoperiod effect on immune status. Housing density of birds per pen was targeted for 30 kg/m^2, which was achieved by placing 63 females and 53 males per pen. Fresh, clean wheat straw litter was used after cleaning and sanitizing the rooms. Chicks were not vaccinated for *Eimeria* at the hatchery, however, anti-cocciodials (Salinomycin sodium @ 60 mg/kg) and an antibiotic (Bacitracin Methylene Disalicylate @ 4.4 mg/kg) were included as feed additives in the experimental diets.

The relative organ weight was assessed only at 36 d in experiment 3 and weekly (12, 19, 26, 34 d) in experiment 4 by randomly selecting one bird per pen (n=108) across

all 12 pens in each room and from a total of 9 rooms. Blood was collected for heterophil-lymphocyte ratio (34-36 d) using the procedure described previously (section 2.3.2.1). Antibody titer and whole-blood chemiluminescence (WBCL) assay was conducted as described below:

2.3.2.2.1 Antibody Titer

Intramuscular administration of 0.2 mL *E. coli* toxoid (Litterguard LT (Pfizer, NY, USA) was used to induce an antibody reponse. In experiment 3 one female bird per pen (n=54) was randomly selected and injected with 0.2 mL of this vaccine at 10 d and 21 d of age, and 1 mL of blood was collected for primary and secondary humoral antibody titer at 21 and 36 d of age, respectively. The same procedure was adopted for male birds (n=54) in experiment 4, where the first injection was given at 10 d and the second booster injection given at 21 d. One mL blood was collected at 21 and 27 d for primary and secondary humoral antibody titer. Collected blood samples were then centrifuged after coagulation at room temperature for 16 h to remove the serum. Antibody titer to crude F4 fimbrae (K88) protein, purified as described by Danabassis (2006), in serum was determined by ELISA. Briefly, the *E.coli* (K88) isolate was cultured overnight at 37 °C in tryptone soy broth (Difco Laboratories, USA). Bacteria were then collected by centrifugation (4,096 x g, 15 min), washed twice and re-suspended in phosphate-buffered saline (PBS, 150mM, pH 7.4). Using a Polytron T45/2 homogenizer, the F4 fimbriae was isolated in supernatant after homogenizing (24,000 rpm, 20 min, 4 °C) and centrifugation for 20 min at 8,074 x g and again at 16,846 x g for 40 min of bacteria culture. The supernatant containing solubilized fimbriae was then transferred and precipitated with 40 % ammonium sulfate for 18 h. The precipitate then obtained was centrifuged (16,846 x g, 40 min), suspended in Tris-HCl (10mM, pH 7.4) plus 2M urea and dialyzed overnight into PBS to produce crude F4 protein. Further, to determine protein content of crude F4 protein, a serial dilution of bovine serum albumin (MJS Biolynx Inc., Canada) standards was prepared and compared. Then, 96-well polystyrene microtiter plates (Immunolon®, USA) were coated and incubated overnight at 4 °C with 25 µg/mL of F4 protein diluted in PBS (Wilkie, 2006). Plates were then blocked with 1 % BSA in PBS-T (0.05 % Tween-20) and washed four times with

PBS-T. Then 100 µL of sample dilution in PBS-T were added to plate and incubated for 1 h at 37 °C with shaking. The plate was washed (4X, PBS-T) and incubated (37 °C, 1 h) with 100 µL of a horseradish peroxidase-conjugated mouse anti-chicken IgG antibody (Sigma) diluted 1:1000 with PBS-T. The plate was again washed (4X, PBS-T) and incubated with 50 µL of a solution consisting of 1 mg/mL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Boehringer Mannheim Biochemica, Germany) substrate for 10 min. The absorbance of each well was then determined at 405 nm using an automated spectophotometer (Molecular Devices, Vmax Kinetic Microplate Reader, USA).

2.3.2.2.2 Whole Blood Chemiluminescence (WBCL) Assay

The whole-blood chemiluminescence (WBCL) procedure was a modification of Papp et al. (2009). Briefly, one mL blood was collected in heparinized tubes and then 50 µL of the sample was diluted (1:5 ratio) in Hank's Balanced Salt Solution (HBSS, Sigma). This mixture was added to a 96-well Polysorp microplate (Nunc, Denmark). Phagocytes were then stimulated by adding 50 µL of Zymosan A from *Saccharomyces cerevisiae* (Sigma) diluted in HBSS to a final concentration of 0.05 mg/mL. Next, 50 µL of 0.05 mM working solution of Lucigenin (N, N0-dimethyl-9,90-biacridium dinitrate; Sigma) in HBSS, a light enhancer, was added to the plate by using a multi-channel pipettor. Plates were shaken for 5 s at 39 ºC before reading with a Fluoroskan Ascent FL machine (Thermo Labsystems). Light emission results were presented as counts per minute (cpm).

2.3.3 Incidence of Infectious and Total Mortality

Dead and cull birds were recorded twice daily for all experiments during this study. Mortality results from first two experiments (1 and 2) has already been published (Schwean-Lardner et al., 2013). The mortality data from experiment 3 and 4 was categorized into 2 groups based on etiology (Schwean-Lardner et al., 2013): infectious mortality caused by infectious agents (such as arthritis, polyserositis, peritonitis and osteomyelitis) and total mortality (all types of mortalities inclusive of infectious mortality).

All mortalities and culls were necropsied for cause of death or morbidity by an independent laboratory, Prairie Diagnostic Services Inc.

2.3.4 Statistical Analyses

Statistical analyses of the collected data (organ weight, H:L ratio, antibody titer, WBCL assay, mortality incidence) was conducted for main effects (photoperiod, sex, and/or genotype, diet) and their interactions, by one-way ANOVA using the Proc GLM procedure of SAS (SAS 9.1, Cary, NC) using individual birds as the replicate unit for photoperiod program and pens nested within room. Percent relative organ weights and mortality data were normalized by log transformation prior to statistical analysis. Additionally, PROC REG and PROC RSReg in SAS (9.1) were used to analyze the relationship between incidence of mortality (calculated on the basis of percent birds initially placed in each pen) and photoperiod or dietary amino acid levels. Significant differences were declared when a P-value of 0.05 or less was recorded.

2.4 Results

2.4.1 Relative Organ Weights

Exposure to 23L in all four experiments and at all ages decreased relative liver organ weight (Tables 2.2 and 2.3), although the effect was significant only in experiment 4 at 26 d of age. In experiments 1 and 4 (Tables 2.2 and 2.3), within one week of photoperiod treatment allocation (12-14 d of age), the lowest spleen weights were noticed in broiler chickens raised either under 13L or 14L when compared to photoperiod treatments providing ≥ 17L exposure. In contrast, after 19-21 d of age, birds exposed to ≤ 17L photoperiod had a higher relative bursa weight, which was significant in experiment 1 only. Across all four experiments female birds revealed a significantly heavier relative liver weight from 14 to 48 d of age (Tables 2.2 and 2.3), while reduced percent relative bursal weight was recorded in older birds (26-48 d of age). The effect of genotype on immune status of broiler chickens, as examined in experiments 1 and 2, revealed a greater percent relative liver weight and a reduced spleen weight in Ross 308 compared to Ross

708 birds between 4 and 5 weeks of age, however this difference was only significant in experiment 1. Moreover, feeding low, compared to medium and high amino acid based diets, promoted higher percent relative liver weights between 19-34 d of age, reaching significance only in experiment 4 (Table 2.3).

Few significant interactions between main effects were observed for organ weight data. At all investigated ages (14, 35, 48 d) in experiments 1 and 2 male broiler birds exposed to 23L had significantly smaller relative liver weight. On contrary, in female broilers the liver weight changes varied with age, with the lowest relative liver weights detected in 48 d old female birds exposed to under 23L (Figure 2.1). Exposure of Ross 308 birds to 23L photoperiod in experiment 1 resulted in greater relative liver weight at 35 d of age (Figure 2.2a). In experiment 2 the relative bursa weight in Ross 308 birds at 48 d age was reduced (Figure 2.2b) versus Ross 708 counterparts. On other hand, in experiment 3, the lowest relative liver weight (Figure 2.3) was recorded in broilers fed high amino acid diets, compared to low and medium diets, when exposed to 23L photoperiod versus ≤ 17L photoperiod regimes.

Table 2.2. Effect of photoperiod, genotype and sex on relative organ weights (g/g bwt) in broiler chicken experiments at selected ages (n=160).

Experiment		Photoperiod (P)[1]				Genotype (G)[2]		Sex (S)[3]		P-value				SEM
		14L	17L	20L	23L	308	708	M	F	PXG	GXS	PXS	PXGXS	
1	14 d													
	Liver	3.82	3.63	3.71	3.60	3.73	3.65	3.59^{b}	3.79^{a}	NS	NS	0.04	NS	0.038
	Spleen	0.08^{b}	0.09^{a}	0.10^{a}	0.10^{a}	0.09	0.09	0.09	0.10	NS	NS	NS	NS	0.002
	Bursa	0.19	0.19	0.19	0.20	0.19	0.20	0.19	0.19	NS	NS	NS	NS	0.004
	21 d													
	Liver	3.09	3.20	3.06	2.90	3.10	3.02	2.99^{b}	3.13^{a}	NS	NS	NS	NS	0.027
	Spleen	0.09	0.08	0.09	0.08	0.09	0.09	0.08	0.09	NS	NS	NS	NS	0.002
	Bursa	0.22^{a}	0.21^{a}	0.20^{ab}	0.18^{b}	0.20	0.21	0.20	0.20	NS	NS	NS	NS	0.004
	28 d													
	Liver	2.69	2.58	2.62	2.54	2.66^{a}	2.56^{b}	2.56	2.62	NS	NS	NS	NS	0.020
	Spleen	0.09	0.10	0.09	0.10	0.10	0.10	0.10	0.10	NS	NS	NS	NS	0.002
	Bursa	0.22	0.21	0.20	0.20	0.21	0.20	0.21	0.20	NS	NS	NS	NS	0.004
	35 d													
	Liver	2.50	2.51	2.48	2.43	2.54^{a}	2.42^{b}	2.43^{b}	2.53^{a}	0.002	NS	0.01	NS	0.023
	Spleen	0.09	0.09	0.10	0.09	0.09^{b}	0.10^{a}	0.09	0.09	NS	NS	NS	NS	0.002
	Bursa	0.20	0.18	0.18	0.17	0.18	0.19	0.19^{a}	0.17^{b}	NS	NS	NS	NS	0.003
2	48 d													
	Liver	2.33	2.24	2.27	2.13	2.27	2.22	2.18^{b}	2.31^{a}	NS	NS	0.02	NS	0.022
	Spleen	0.11	0.09	0.09	0.11	0.10	0.10	0.10	0.10	NS	NS	NS	NS	0.003
	Bursa	0.16	0.15	0.13	0.15	0.14	0.15	0.16^{a}	0.14^{b}	0.04	NS	NS	NS	0.003

[1]P; 14L=14L:10D, 17L=17L:7D, 20L=20L:4D, 23L=23L:1D; [2] G; 308=RossXRoss 308, 708=RossXRoss 708, [3] S; M=Male, F=Female.

[a,b]Means with the same letter are not significantly different in the same column. P-values were considered significant at P<0.05.

Table 2.3. Effect of photoperiod, diet and sex on relative organ weights (g/g bwt) in broiler chicken experiments at listed age (n=108).

Experi-ment		Photoperiod (P)[1]			Diet (D)[2]			Sex (S)[3]		P-value				SEM
		15L	17L	23L	Lo	Med	Hi	M	F	PXD	DXS	PXS	PXDXS	
3	36 d													
	Liver	2.52	2.53	2.47	2.58	2.52	2.42	2.40[b]	2.61[a]	0.002	NS	NS	NS	0.041
	Spleen	0.10	0.10	0.12	0.10	0.11	0.10	0.11	0.10	NS	NS	NS	NS	0.003
	Bursa	0.15	0.16	0.16	0.15	0.15	0.16	0.17[a]	0.14[b]	NS	NS	NS	NS	0.005
4		13L	18L	23L										
	12 d													
	Liver	3.92	3.93	3.84	4.08	3.84	3.77	3.85	3.95	NS	NS	NS	NS	0.052
	Spleen	0.07[b]	0.08[a]	0.08[a]	0.08	0.08	0.08	0.08	0.08	NS	NS	NS	NS	0.002
	Bursa	0.19	0.19	0.20	0.19	0.19	0.19	0.20	0.18	NS	NS	NS	NS	0.005
	19 d													
	Liver	3.46	3.30	3.09	3.41[a]	3.20[b]	3.24[b]	3.24	3.33	NS	NS	NS	NS	0.044
	Spleen	0.07	0.08	0.09	0.08	0.08	0.08	0.08	0.08	NS	NS	NS	NS	0.002
	Bursa	0.24	0.23	0.23	0.22	0.23	0.23	0.24	0.22	NS	NS	NS	NS	0.006
	26 d													
	Liver	3.08[a]	2.89[b]	2.78[b]	3.12[a]	2.83[b]	2.78[b]	2.88	2.95	NS	NS	NS	NS	0.037
	Spleen	0.09	0.09	0.10	0.09	0.09	0.09	0.09	0.09	NS	NS	NS	NS	0.002
	Bursa	0.22	0.21	0.20	0.21	0.22	0.20	0.22[a]	0.19[b]	NS	NS	NS	NS	0.004
	34 d													
	Liver	2.83	2.71	2.70	2.86[a]	2.72[b]	2.67[b]	2.69[b]	2.81[a]	NS	NS	NS	NS	0.033
	Spleen	0.09	0.10	0.11	0.11	0.09	0.10	0.11	0.10	NS	NS	NS	NS	0.003
	Bursa	0.22	0.20	0.18	0.20	0.19	0.21	0.21	0.19	NS	NS	NS	NS	0.006

[1]P; 13L=13L:11D, 15L:3.5D:2L:3.5D, 17L=17L:7D, 18L=18L:6D, 23L=23L:1D ; [2]D; Lo=Low, Med= Medium, Hi=High; [3] S; M=Male, F=Female. [a,b]Means with the same letter are not significantly different in the same column. P-values were considered significant at $P<0.05$.

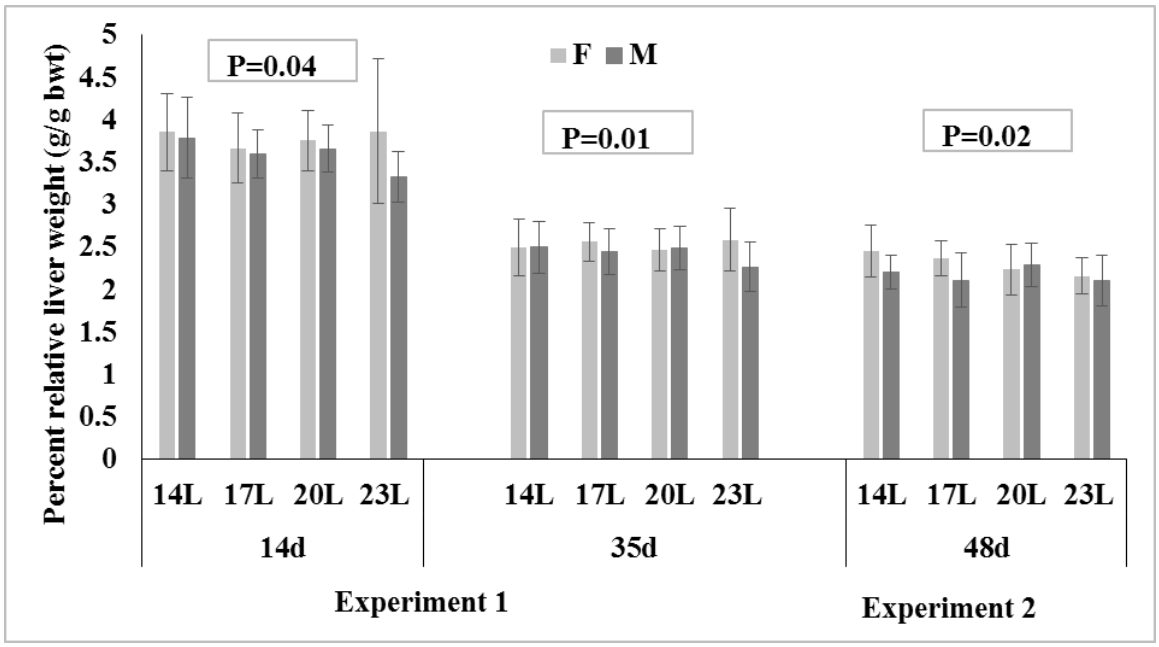
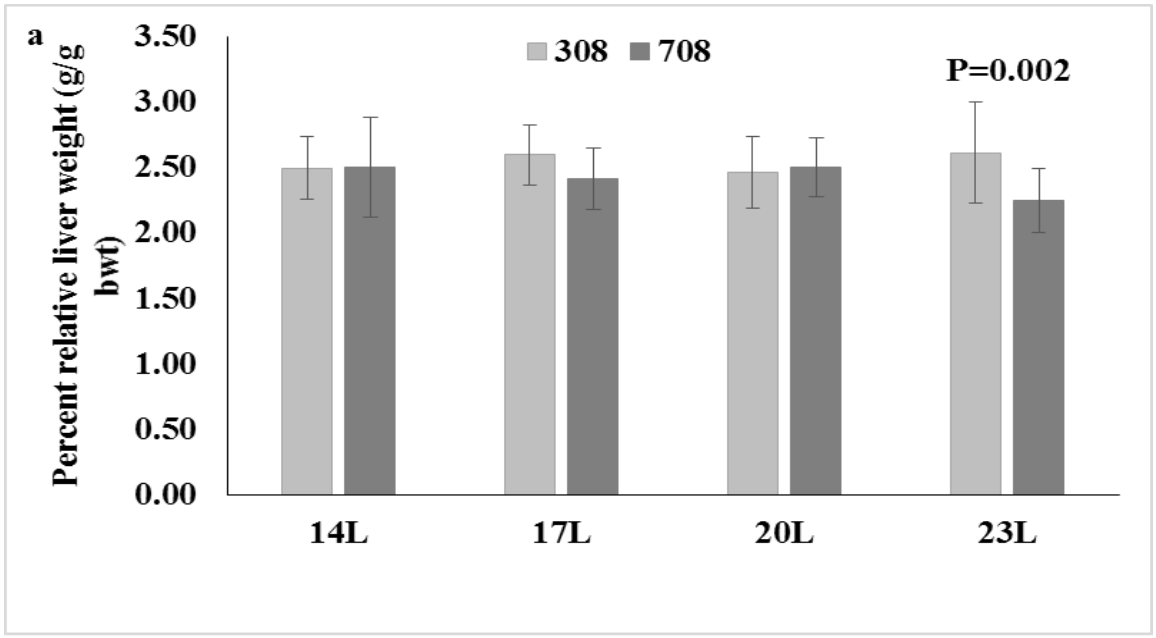


Figure 2.1. Interaction of photoperiod and sex on percent relative liver weight assessed in broiler chicken experiment 1 and 2.



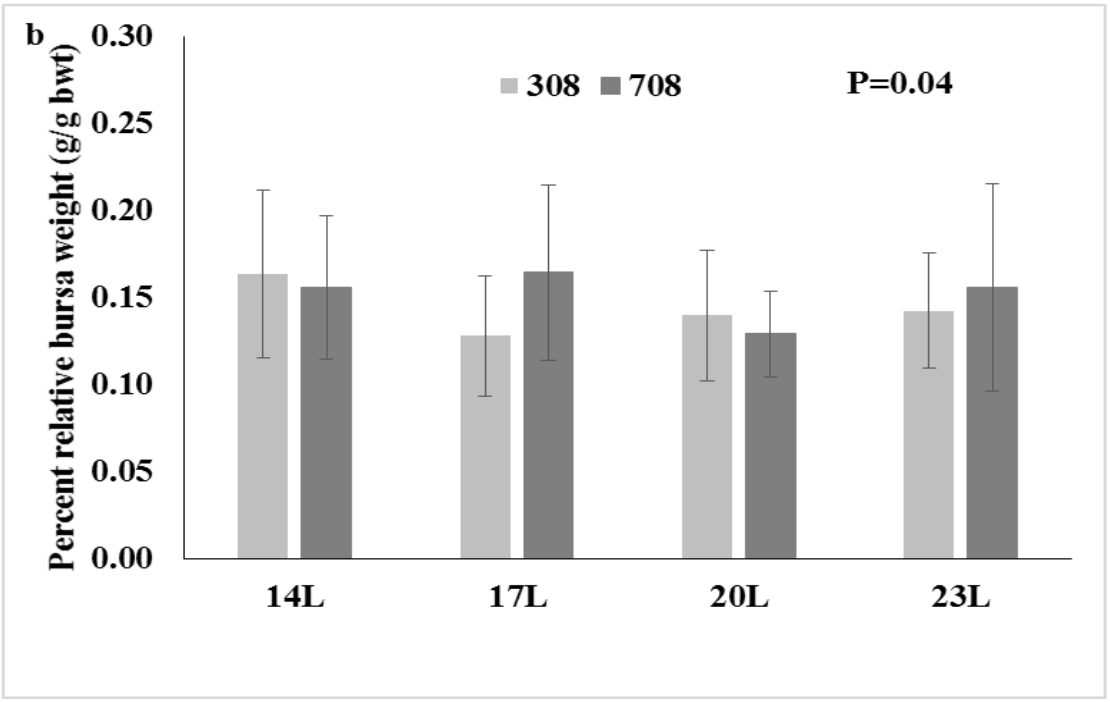


Figure 2.2. Interaction of photoperiod and genotype on percent relative (a) liver and (b) bursa weights assessed in broiler chicken experiment 1 and 2.

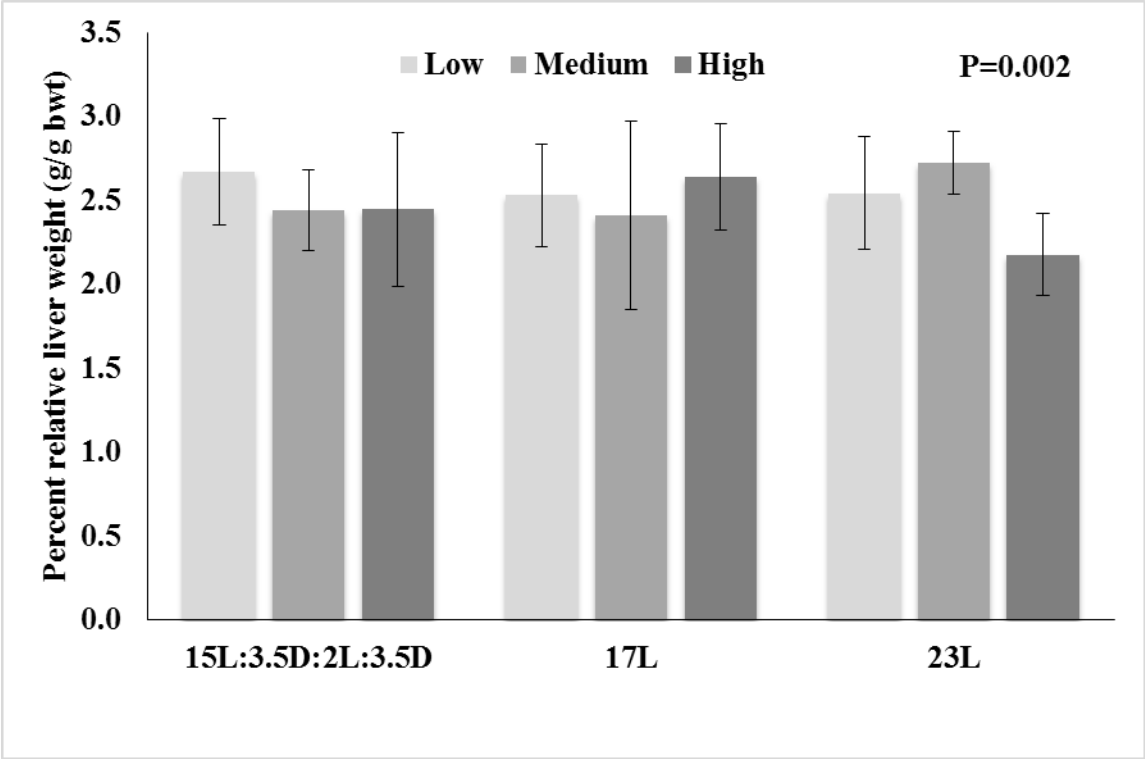


Figure 2.3. Interaction of photoperiod and diet fed on percent relative liver weights assessed in broiler chicken experiment 3.

2.4.2 Chronic Inflammatory Challenge

In experiments 1 and 2 chronic LPS challenge negatively influenced weight gain and final body weight (Table 2.4) at the end of the ten-day challenge period. However, the effect was significant only in the first experiment. Challenge with LPS increased relative weight of liver (experiment 1, P=0.09; experiment 2, P<0.001) and spleen (P <0.001) in both experiments. No effect of challenge on bursa weight or H:L ratio was observed. Photoperiod significantly affected relative bursa weight in birds injected with saline (control), however, the effect was not consistent between the two experiments where bursa weight was highest in birds exposed to 14L in experiment 1 compared to 17L in experiment 2. A significant effect of photoperiod was observed on the H:L ratio in experiment 1 despite highly variable results. In general, H:L ratio was elevated in birds exposed to 17L or

greater. No interaction was observed between photoperiod and LPS challenge for any of the parameters analyzed during experiments 1 and 2 indicating that photoperiod did not alter responses to inflammatory challenge.

Table 2.4. Parameters evaluated in Lipopolysaccharide (LPS) challenged birds (Ross 308) in experiment 1 and experiment 2 raised under 4 different photoperiod (P)[1] regimes (n=64).

Experi-ment	Parameter	Day	14L		17L		20L		23L		P-value		
			Control	LPS	Control	LPS	Control	LPS	Control	LPS	LPS	P	LPS X P
1	Body weight	26	1198	1180	1326	1233	1355	1344	1371	1256	<0.05	0.11	0.17
	Gain	17-26	658	630	727	652	736	702	756	658	<0.01	0.24	0.19
	Liver	26	2.86	2.76	2.56	2.70	2.51	2.63	2.61	2.82	0.09	0.31	0.18
	Spleen	26	0.09	0.12	0.09	0.11	0.09	0.12	0.09	0.12	<0.01	0.90	0.83
	Bursa	26	0.24	0.25	0.22	0.23	0.21	0.22	0.22	0.22	0.48	0.01	0.97
	H:L ratio	26	0.50	0.67	1.94	3.64	3.99	2.16	1.15	1.18	0.95	0.04	0.17
2	Body weight	36	2327	2256	2312	2223	2480	2405	2259	2179	0.42	0.11	0.43
	Gain	27-36	889	848	813	798	862	823	751	673	0.13	0.02	0.56
	Liver	36	2.24	2.56	2.35	2.58	2.37	2.67	2.27	2.49	<0.01	0.45	0.87
	Spleen	36	0.12	0.16	0.11	0.12	0.11	0.13	0.11	0.14	<0.01	0.37	0.07
	Bursa	36	0.18	0.19	0.22	0.19	0.18	0.17	0.19	0.19	0.18	0.012	0.36
	H:L ratio	36	0.47	0.53	0.93	1.32	0.78	0.78	1.56	0.63	0.58	0.38	0.17

[1]P= Photoperiod length (L) per day.

2.4.3 Antibody Titer

For both experiments 3 and 4, the effects of photoperiod, diet and sex on primary (data not shown here) and secondary antibody titer response were not significant (Table 2.5). Moreover, no interactions were observed between investigated main effects.

2.4.4 Heterophil: Lymphocyte Ratio

Consistently in both experiment 3 and 4 (Table 2.5) the proportion of heterophils and lymphocytes in blood was not affected by photoperiod, diet or gender of broilers. However, the highest heterophil counts and H:L values were detected under 17L or 23L, while broiler chickens raised under either 15L or 13L demonstrated the lowest H:L ratio and heterophil counts.

2.4.5 Whole Blood Chemiluminescence (WBCL) Assay

No effect of photoperiod, diet and sex was observed on the WBCL assay conducted either at 36 d of age in experiment 3 or weekly in experiment 4 (Table 2.5). The exception was in experiment 4, where feeding of a high amino acid diet improved the heterophil phagocytosis index in 34 d old broiler chickens compared to their low and medium crude amino acid diet fed counterparts.

2.4.6 Incidence of Infectious and Total Mortality

In experiment 4 (Table 2.5) the incidence of total and infectious mortality (%) analysed from 7-36d increased as photoperiod length increased, as evidenced by a significant linear regression (total mortality, P=0.008, and infectious mortality, P=0.053). The highest infectious mortality values were observed in broilers exposed to 23L photoperiod in contrast to 13L and 18L photoperiod regimes. For both experiments 3 and 4 (Table 2.5) the incidence of mortality calculated on the basis of percent birds initially placed in each pen revealed that dietary amino acid levels did not affect infectious and total mortality incidence. In experiment 3 significantly higher total mortality incidence was recorded in male broiler chickens compared to female birds. Although this was not

significant in experiment 4, still males had higher total mortality values in comparison to their female counterparts.

Table 2.5. Effect of photoperiod, diet and sex on antibody titer, Heterophil:Lymphocyte (H:L) ratio, Whole Blood Chemiluminescence (WBCL) and mortality incidences in broiler chickens.

Experi-ment	Variable and age of analysis	Photoperiod (P)[1]			Diet (D)[2]			Sex (S)[3]		P-value				SEM
		15L	17L	23L	Lo	Med	Hi	M	F	PXD	DXS	PXS	PXD XS	
3														
	Antibody titer (36 d)	3.68	3.93	4.16	3.83	3.95	3.98	3.95	3.89	NS	NS	NS	NS	0.056
	Heterophil (36 d)	21.54	24.51	22.58	23.53	22.37	22.69	22.16	23.60	NS	NS	NS	NS	0.680
	Lymphocyte (36 d)	38.46	35.49	37.42	36.47	37.63	37.31	37.84	36.40	NS	NS	NS	NS	0.680
	H:L ratio (36 d)	0.59	0.77	0.69	0.74	0.65	0.66	0.65	0.72	NS	NS	NS	NS	0.038
	WBCL (cpm) (36 d)	4244	6550	4571	5100	5863	4312	---	---	NS	---	---	---	583.7
	Mortalities[4] (7-37 d)													
	Infectious	1.04	1.07	0.87	0.65	1.02	1.32	1.22	0.76	NS	NS	NS	NS	0.136
	Total	5.50	4.40	5.04	4.74	4.66	5.53	6.99[a]	2.96[b]	NS	NS	NS	NS	0.353
4		13L	18L	23L										
	Antibody titer (27 d)	3.49	3.54	3.49	3.47	3.49	3.55	3.56	3.46	NS	0.02	NS	NS	0.045
	Heterophil (34 d)	21.27	24.00	28.89	25.22	22.67	26.28	24.59	24.85	NS	NS	NS	NS	1.100
	Lymphocyte (34 d)	38.72	36.00	31.11	34.78	37.33	33.72	35.41	35.15	NS	NS	NS	NS	1.100
	H:L ratio (34 d)	0.60	0.83	1.04	0.84	0.71	0.92	0.83	0.81	NS	NS	NS	NS	0.079
	WBCL (cpm)													
	12 d	4694	4524	5139	4678	4965	4739	4630	4958	NS	NS	NS	NS	132.8
	19 d	5089	5213	5263	5706	4800	5038	5535	4804	NS	NS	NS	NS	248.4
	26 d	4117	5522	6211	5739	4983	5128	5926	4641	NS	NS	NS	NS	508.5
	34 d	5739	5700	5844	5617[ab]	4983[b]	6683[a]	5682	5841	NS	NS	NS	NS	225.9
	Mortalities (7-38 d)													
	Infectious*	0.48	0.73	1.10	0.78	0.84	0.68	0.98	0.56	NS	NS	NS	NS	0.129
	Total**	0.92	1.38	2.23	1.67	1.34	1.48	1.93	1.09	NS	NS	NS	NS	0.205

[1]P; 13L=13L:11D, 15L:3.5D:2L:3.5D, 17L=17L:7D, 18L=18L:6D, 23L=23L:1D ; [2] D; Lo=Low, Med= Medium, Hi=High;
[3] S; M=Male, F=Female
[4]Mortalities were calculated % of birds placed per pen
*Regression equation for Infectious mortality : Y=-0.34 + (0.06x), P value=0.053
**Regression equation for Total mortality: Y= -0.86 + (0.13x), P value=0.008.

2.5 Discussion

Photoperiod duration has been described as the predominant zeitgeber which can hugely affect broiler chicken growth, immune status and livability (Schwean-Lardner et al., 2016). Poultry birds usually eat during the photophase, therefore, it is not uncommon to believe that post-hatch installation of long photoperiods in commercial broiler production system might result in superior performance (Schwean-Lardner et al., 2016). However, considerable evidences indicate a suppressing effect of long photoperiods, allowing only a short or no dark period exposure, on the broiler chicken immune system as assessed by several established innate and acquired immune responses (Gordon 1994; Onbaşılar et al., 2007; Abbas et al., 2008; Lewis et al., 2009; Abbas, 2013; Zheng et al., 2013; El Sabry et al., 2015). Unfortunately, due to lack of consistent broiler immune status data and interpretation bias, the effects of photoperiod on immuno-competence and infectious mortality are not clearly outlined and are inconclusive (Prendergast et al., 2003; Onbaşılar et al., 2007; Davis et al., 2008; Schwean-Lardner et al., 2013). Therefore, in the current study, several innate and acquired immune responses, along with the incidence of mortality, across different photoperiod regimes were investigated to aid in the assessment of the role of photoperiod on the immune status of broilers raised under commercial and challenged states.

The present study in general indicated no influence of photoperiod on broiler chicken relative organ weights, which is in agreement to Onbaşılar et al. (2007), Guo et al. (2010) and El Sabry et al. (2015). In contrast to our hyposthesis, the smallest relative spleen weight was recorded in birds exposed to 13L/14L, compared to photoperiod schedules (17L-23L) at 14 d of age, one week following implementation of photoperiod regimes in experiment 1 and 4, suggesting these birds were immunologically stressed. Since all birds were exposed to 23 L from 0-7 d of age, initial long dark periods of $\geq$ 6 h may have caused stress due to starvation, leading to increased plasma corticosteroid concentration (Abbas et al., 2008) and possibly immunosuppression. After this age (12-14 d) in all experiments revealed no effect of photoperiod on relative weight of the spleen agreeing with previous

reports in broiler (El-Sagheer et al., 2004; Onbaşılar et al., 2007). However, reduced relative bursa and liver weights were consistently recorded under the 23L photoperiod, although only rarely were these differences statistically significant ($P \leq 0.05$). Earlier findings have (Panshikar and Haldar, 2009; Zheng et al., 2013) suggested increased darkness exposure and heavier organ weights representing enhanced immune status. However, inconsistency and dilemma still persist regarding effect of photoperiod on organ weights, hence, organ weight results should be carefully interpreted. Increase in organ size may not necessarily be associated with a better immune response at all times, as it can indicate an ongoing infection or parasitic load or stress (Smith and Hunt 2004). For instance, increased liver weights recorded here in birds reared under 14L do not necessarily indicate better immune status, as it could also be due to excess fat storage, plasma corticosteroid (Abbas et al., 2008) or altered rate of metabolism due to extended dark period exposure (Puvadolpirod and Thaxton, 2000; Samms et al., 2014). Investigations by Puvadolpirod and Thaxton (2000) revealed liver hypertrophy due to fat deposition and moisture depletion in broilers under corticosteroid-induced stress. Likewise, Samms et al. (2014) have identified differential liver fat storage or metabolism patterns in hamsters raised under 8L:16D compared to 16L:8D, along with reduced activity and metabolism to cope with the nocturnal fast. We did not investigate the chemical composition of liver tissue in the current study making interpretation of relative liver weight on the metabolic or immune status of the bird difficult.

Bursa weights increased with increased darkness exposure which agrees with Zheng et al. (2013) who investigated photoperiod effects (24L:0D, 16L:2D:1L:2D:1L:2D and 17L:3D:1L:3D) on broiler chicken bursa and thymus weights over 1-50 d period. Furthermore, compared to liver weight which can have confounding effects due to metabolism changes (Samms et al., 2014), bursa is regarded as the main lymphoid organ involved in immune response generation (Zheng et al., 2013). Moreover, bursa mass has been suggested to be a more accurate indicator of immune cells involved in immune response generation (Fox and Grasman, 1999). Poultry organ weights have been used as common indicator for immune status in chickens (Heckert et al., 2002; Onbaşılar et al.,

2007), as they can be effortlessly recorded, reflecting the body's capability to impart lymphoid cells during an immune response. Low weight can be associated with immune stress and adversely affect host capabilities (Prendergast et al., 2003; Panshikar and Haldar, 2009; El Sabry et al., 2015). Therefore, it can be concluded that increased photoperiod reduced bursa weights here, which might suggest an immune stress.

Inconsistent effects of genotype and sex on relative organ weights were also recorded in this study; for instance, female birds had increased liver and decreased bursa weight. Feeding low amino acid diets also resulted in heavier liver, which agrees with earlier findings in broilers where feeding suboptimal protein diets increased immune responses, bursa weights (Guo et al., 2010) and immunoglobulin synthesis (Ferreira et al., 2016). It was suggested that hosts with an enhanced immune response can redirect flow of dietary nutrients to support immune cells or molecules synthesis, which however could lead to production losses (Ferreira et al., 2016).

Immune and growth indices evaluated after chronic LPS challenge indicated decreased performance and increased immune stress, which agrees with previous reports (Korver et al., 1998; Webel et al., 1998; Xie et al., 2000; Liu et al., 2015). The decreased body weight and gain observed after chronic endotoxin challenge in both experiments (1 and 2) can be attributed to the general catabolic state typically observed during an acute inflammatory response (Liu et al., 2015) as observed previously with similar LPS challenge models (Webel et al., 1998). Although this response was not significant in experiment 2, the bacterial endotoxin challenge still depressed final body weight and gain of LPS-injected birds versus their saline injected counterparts. In agreement with previous findings (Roura et al., 1992; Mireles et al., 2005), relative organ (liver and spleen) weights were consistently elevated by LPS challenge, suggesting increased immune cell abundance in response to inflammatory challenge (Korver et al., 1998; Prendergast et al., 2003). Liver is the main supplier of acute phase protein in case of inflammatory response and is typically assessed by relative liver size analysis (Korver et al., 1998). Since B-cells are not involved in this type of challenge, lack of response of challenge on bursa weights was anticipated. Ratios of H:L were also not significantly affected by LPS challenge. This agrees with Xie

et al. (2000) who studied H:L ratios in three-week old broilers 12, 24 and 40 h post *Salmonella typhimurium* LPS injections. Xie et al. (2000) found only significant elevation of H:L ratios 12 h post LPS injection, with ratios returning to control values within 24 h, suggesting early influx of heterophils in circulation after LPS injection, which later move from blood to the site of inflammation (Harmon, 1998; Shini et al., 2008). Unexpectedly high H:L ratios (> 3) were noticed in experiment 3 in birds on 17L and challenged with LPS and in birds on 20L and injected with saline. We speculate this could be due to stress from additional handling, repeated injections and blood sampling (Gross and Siegel, 1983) over the 10 d challenge period. This also suggests that LPS alone did not further elevate the stress response beyond that of handling. Furthermore, no consistent interactions were detected for investigated variables between photoperiod and LPS, which contradicts Fonken et al. (2012) and Prendergast et al. (2003) who showed that LPS challenge in Siberian hamsters housed under short photoperiod exposure (8L:16D) resulted in reduced physiological and behavioral symptoms in comparison to 16L:8D photoperiod exposed littermates. In agreement, (Gehad et al., 2008) exposure of day old male broiler chickens to intermittent (1L:3D) photoperiod schedule enhanced immune responses (total WBCs), reduced plama corticorteriod levels and body temperature when injected with LPS (3 mg/kg bwt) at 6-week of age versus 23L:1D exposed birds. This difference was suggested due to shorter photoperiod induced melatonin production and its direct and indirect roles in host immune status (Prendergast et al., 2003; Gehad et., 2008; Fonken et al., 2012). Another reason for the observed inconsistencies after LPS challenge may be the multiple LPS injections over a short period (10 d). Such multiple LPS injections may act as a chronic stressor, thereby decreasing the magnitude of the inflammatory responses generated, in comparison to the use of a single injection. In support, Takahashi et al. (1995) and Korver et al. (1998) reported in their chicken-LPS work that second or repeated LPS injections were not as effective as the first injection, possibly due to immune intolerance development (Kogut, 2009).

The H:L ratio has been used an established indicator of stress in several broiler chicken experiments (Gross and Siegel, 1983; Heckert et al., 2002; Abbas et al., 2008)

where elevated values have been proposed to reflect stressed environmental conditions due to constant exposure to 23L:1D/24L:0D photoperiod (Campo et al., 2007; Coban et al., 2014) compared those allowing greater than 8 h of darkness. Although non-significant and coherent to Lien et al (2007) work, nonetheless basal H:L values recorded in the current study in birds exposed to ≥17L photoperiod regimes in two separate experiments (Table 2.5) were close or exceeded the high stress values of 0.60, suggested by Gross and Siegel, (1983). However, literature regarding H:L ratios has contradictory findings, when compared 24L/23:1D photoperiod schedules had no significant influence on H:L ratios versus increasing (Wang et al., 2008) or 1L:3D (Onbaşılar et al., 2007) photoperiod regeimes allowing more darkness exposure. On contrary, few reported highest H:L ratios values when birds were exposed to long (23L:1D) (Campo and Dávila, 2002; Blair et al., 1993; Lien et al., 2007) or restricted (12L:12D) photoperiod (Abbas et al., 2008) schedules. Although, the absence of an effect of photoperiod on H:L ratios does not necessarily always indicate that the host is not stressed. As Puvadolpirod and Thaxton (2000) have proposed, the stress response may vary during initial and later stages of life, and even may completely fade off. They found elevated H:L ratios detectable only to 10 d post-induction of the stress condition. As blood samples for H:L ratios were collected 3-4 weeks after introduction of the photoperiod regimes, it may be possible that the photoperiod effect on H:L ratio may have disappeared over time. Especially for H:L ratios, during immune stress initial high influx of heterophils has been reported into peripheral circulation (≥12 h), which later migrate to site of inflammation (Harmon, 1998; Shini et al., 2008) resulting in lowering H:L ratio in blood over next 24-48 h period. However, not measured here in current work, increased photoperiod may increase plasma corticosteroid amount (Lien et al., 2007), chronic exposure of which can further downregulate immune response, as reduction in H:L ratios have been repoted 10 d post corticosterone treatment (Shini et al., 2008).

Our findings for antibody development following vaccination with *E. coli* toxoid agrees with Onbaşılar et al. (2008), where photoperiod (16L:8D versus 24L:0D) did not influence antibody titers to sheep red blood cells (SRBC) and New Castle Disease virus (NDV) vaccination evaluated in 36 d-old broiler chickens. However, contrastingly,

significantly higher antibody titers against NDV (Onbaşılar et al., 2007), SRBC (Abbas et al., 2008) and bovine serum albumin (Abbas, 2013) have been reported in broilers raised under intermittent photoperiod regimes, when given a greater amount of darkness compared to their continuous lit counterparts. The absence of a dark period in continuous photoperiod reared male birds was postulated as a reason for the depressed ability to raise antibody tires (Onbaşılar et al., 2007). Likewise, implementing longer darkness (12L:12D) to 6 weeks old male broiler chickens has revealed similar antibody production against SRBC, compared to 23L:1D photoperiod containing short dark period (Abbas et al., 2008). They suggested the negative effect on antibody production in the broiler chickens exposed to extended darkness was due to higher plasma corticosterone levels observed in their experiment (Abbas et al., 2008), probably due to feed restriction. A clear reason for this discrepancy was difficult to speculate in present work, as feed restriction in broiler chicken, has also been indicated for positive effect on humoral and cellular immune response compared to *ad-lib* fed counterparts (Khajavi et al., 2003)

Photoperiod did not affect heterophil functionality in the current study, in contrast to others who have observed significantly enhanced monocyte phagocytosis index in birds exposed 6 h of intermittent darkness per day in comparison to a 24L:0D photoperiod, (Zheng et al., 2013). The whole blood chemiluminescence (WBCL) assay measures heterophil phagocytosis and oxidative burst activity, which constitutes a first line of defense against infection (Papp and Smits, 2007; Papp et al., 2009). On contrary, exposure to 8L:16D versus 16L:8D suppressed both phagocytosis of *Staphyloccocus aureus* bacteria and oxidative burst of granulocytes and monocytes of Siberian hamster (Yellon et al., 1999). In their work, it was suggested that photoperiod differently affect immune cells and their functionality. As, short photoperiod exposed hamsters revealed to invest more in less energy demanding immune responses (innate) compared to acquired which are mostly energetically expensive and shortened, primarily as an energy saving host strategy (Yellon et al., 1999). Another reason for insignificant broiler heterophil phagocytosis assay could be due to a relatively low oxidative burst in response to zymosan has been documented in

chicken heterophil, in comparison to human and canine neutrophil activity (Brooks et al., 1996)

Although non-significant in experiment 3, however, both total and infectious mortality in the next experiment (4) revealed a significant increase with photoperiod exposure. In concordance, Valenzuela et al. (2012) has confirmed that implementing an artificial photoperiod makes fish more susceptible to infections and thus infectious mortalities. Concurrently, and in agreement findings here, Schwean-Lardner et al. (2013, 2016) examined the incidence of infectious mortality in broiler chickens during the 7-48 d period and demonstrated that lowest incidences occurred in broilers raised under 14L:10D in comparison to 17L, 20L and 23L regimes. Similarly, in turkeys, the highest levels of mortalities have been recorded after exposure to 23L in comparison to 14L, 17L and 20L (Vermette et al., 2016). Finally, in agreement to Schwean-Lardner et al. (2013), the current study revealed that Ross 308 males have a higher infectious and total percent mortality rates, than their female counterparts. To date, the effect of photoperiod on non-infectious mortalities has been extensively investigated (Hassanzadeh et al., 2003; Schwean-Lardner et al., 2013, 2016; Vermette et al., 2016) indicating inconsistencies regarding the influence of photoperiod (Gordon, 1998; Ingram et al., 2000; Downs et al., 2006; Lien et al., 2007). Although a positive effect of darkness exposure on immunity has often been cited as explaining reduced infectious mortalities (Abbas et al., 2008; Schwean-Lardner et al., 2013, 2016), other mechanisms are also possible. For example, an overall decrease in the incidence of infection mortality under short photoperiod exposure could be due to the use of the crop for food storage (Buyse et al., 1993, Duve et al., 2011), increasing fermentation activity at this location and possibly preventing colonization of pathogenic bacteria (Durant et al., 1999; Hinton et al., 2000).

Overall, improved immune function associated with increased darkness exposure could also be mediated via melatonin secretion (Moore and Siopes, 2000; Kliger et al., 2000; Özkan et al., 2006; Schwean-Lardner et al., 2013). Melatonin secretion can alter host immune response generation directly through melatonin receptors present on several immune tissues, such as on white blood cells (Calvo et al., 1995), or indirectly, through

endocrine hormones (particularly via the thyroid hormone) (Poon et al., 1994). In support, Hassanzadeh et al., (2016) has recently suggested both darkness exposure and dietary melatonin supplementation (40 ppm) of broiler chicken significantly reduced heat stress induced mortalities. On the contrary, adverse effects of constant or near constant photoperiod (23L) exposure could be due to the release of stress hormones, corticosteroids, resulting in decreased broiler chickens immune health (Post et al., 2003; Özkan et al., 2012; Singh et al., 2010).

Avian immune function has a multifactorial basis, and it is probable that adding darkness is only one of several factors capable of improving health and immunity. Experiments 1-4 reported here included a number of experimental design variations that may have affected immune reponse. Indeed, reuse of litter in experiments 1 and 2 was and attempt to better mimic the higher infectious challenge conditions of commercial system. Nevertheless, whether this was achieved or whether used little improved microbiota maturation and thus bird performance is unclear (Lu et al., 2003, Corzo et al., 2007). Coccidiosis vaccination at hatch (Lillehoj and Lillehoj, 2000), genetic differences in immune response generation (Ardia et al., 2011), and dietary exclusion of antibiotic and anticoccidials (Dibner and Richards, 2005) in experiment 1 and 2 might have contributed to altered immune responses.

Additionally, ontogenic, maternal, epigenetics and, hormonal (corticosteroid) influences (Lovland et al., 2004; Ardia et al., 2011) may also further add to the complexity of immune status determination. Recent work in the pig demonstrate possible "programming" effects of environmental changes in the embroyonic and early postnatal period. Piglets from sows kept on 16L:8D during both farrowing and lactation period tended to show decreased total WBC ($P = 0.08$), while on other hand, piglets from sows kept on 8L:16D had higher concanavalin A, lipopolysaccharide-induced proliferation responses and plasma cortisol (Niekamp et al., 2006).

A major challenge in the interpretation of results reported here is with repect to the increase in infectious mortality observed without detection of major changes in immune function. Assuming infectious challenge levels were similar among treatment groups,

differences in infectious mortality are expected to arise from a compromise in immune function. Nevertheless, while we detected minor changes in immune organ weight, no evidence of compromised immune function was detected. Assessment of immune function was performed her on a small subset of birds and thus the statistical power to detect subtle differences in immune function was reduced. Further, while we selected a number of diverse measurements, the abilty of these measurments to fully assess changes in a complex innate and acquired immune response is clear incomplete.

2.6 Conclusions

In conclusion, investigation of broiler chickens' immune status, when kept under range of photoperiod schedules, has indicated that exposure to extended photoperiods (23L) can reduce organ (bursa and liver) weights and increase infectious mortalities. Other measures of innate immune function and inflammatory response were not affected by photoperiod. However, it does not indicate absence of stress as effect of continuous photoperiod on immune response which may be understated in these experiments. Hence, more practical experimental design directly examining effect on diseases or challenge resistance still remains to be investigated. Therefore, further work needs to be conducted in the future to examine and explain the direct correlation of photoperiod on bird immune status, which can be accomplished by evaluating hormones (melatonin and/or plasma corticosterone) and extensive immunologic indicators (cell or humoral mediated immunity), alongside performance and overall livability parameters.

3.0 EFFECT OF PHOTOPERIOD AND DIETARY AMINO ACID CONTENT ON PERFORMANCE, BREAST YIELD AND MICROBIAL ECOLOGY OF THE CROP IN BROILER CHICKENS

3.1 Abstract

Photoperiod induced performance, feed intake, breast meat yield, livability and microbial ecology changes in broiler chickens may affect their dietary requirements for amino acids. Therefore, the objective of the present study was to collectively investigate main effects and interactions between photoperiod and dietary amino acid requirements of broiler chickens, marketed at 39 d of age. From day of placement, birds were randomly assigned to one of three dietary amino acid levels (low, medium, high) and raised under near-continuous photoperiod (23L:1D) exposure. After 7d of age, three selected photoperiod programs; 13L:11D (13L), 18L:6D (18L) and 23L:1D (23L) were initiated. Bird performance and feed intake were recorded weekly until 36 d of age, and carcasses for breast meat yield were processed after being slaughtered at 39 d of age. Relative crop weight and pH were measured at 12, 19, 26, 33 d of bird age. Crop contents were collected at 33 d for analysis of microbial composition using 16S rRNA gene-based terminal-fragment length polymorphism (T-RFLP) and quantitative polymerase chain reaction (qPCR).

The 23L photoperiod exposure resulted in a significantly greater body weight and feed intake, however, feed efficiency was decreased compared to 13L. Feeding of both medium and high dietary amino acid levels supported the greatest body weight and highest feed efficiency. During the experiment, at all investigated time points, males were heavier, ate more and were more feed efficient in contrast to their female counterparts. Total breast meat yield increased with increasing photoperiod exposure, and female broilers demonstrated a higher breast meat yield compared to males.

Overall, the relative crop size decreased with increasing photoperiod duration of ≥18L compared to 13L. The lowest crop pH ($p<0.05$) was recorded for broiler birds reared under 13L at all ages, except initially in 12 d old birds. Moreover, exposure of broilers to 13L photoperiod promoted a greater mean relative abundance of Msp1 TRFs identified as *Lactobacillus* spp. Although there was no effect of photoperiod on diversity indices, cluster analysis of TRFs revealed a grouping based on photoperiod and dietary amino acid levels. Furthermore, the qPCR enumeration of abundant and selected bacterial targets revealed an increase of total bacteria and *Lactobacillus* spp. in the crop of broilers housed under 13L. In conclusion, both increased photoperiod and amino levels increased final body weight and breast meat yield while reduced photoperiod increased feed efficiency and increased microbial abundance in the crop, particularly *Lactobacillus* spp.

3.2 Introduction

Both photoperiod (Schwean-Lardner et al., 2012a, 2013, 2016; Mlaba et al., 2015) and dietary amino acid levels (Hickling et al., 1990; Kidd et al., 2004) have been shown to significantly affect performance, breast meat yield, feed intake and health (assessed by livability) of broiler chickens when raised in environmentally controlled and artificially illuminated buildings. Recently, photoperiod duration has been under extensive review for poultry due to the latest recommendations of the European Council Directives (2007), which link health and welfare concerns with lighting programs utilizing long photoperiods (Abbas et al., 2008; Abbas 2013; Schwean-Lardner et al., 2012 a,b, 2013). The Codes of Practice for broiler production in Canada for photoperiod length, has recently been approved, (http://www.nfacc.ca/codes-of-practice/chickens-turkeys-and-breeders), proposing a minimum of 4 h of continuous darkness to improve bird welfare and health. Coherently, several literature findings indicate a minimum of 6 to 8 h of darkness is required to support performance, immune status, and livability in broilers (Olanrewaju et al., 2006; Schwean-Lardner et al., 2012a,b, 2013) by providing birds adequate rest and reducing stress. Numerous types of photoperiod regimes (short, long, intermittent, intermediate, increasing) are practiced by poultry producers across the world (Olanrewaju et al., 2006; Lewis et al., 2009; Schwean-Lardner et al., 2012a,b; Abbas 2013). However,

a number of studies examining near-continuous (23L:1D) or continuous (24L:0D) photoperiods, widely adopted for maximum growth (Olanrewaju et al., 2006; Schwean-Lardner et al., 2012a, 2016; Hassanzadeh et al., 2016) suggest these programs may indeed fail to provide maximum performance. On other hand, broiler chicken feed intake and breast meat yield has demonstrated a positive association with photoperiod duration (Brickett et al., 2007; Lien et al., 2007; Lewis et al., 2009; Mlaba et al., 2015).

Feed consumption and feeding patterns can be markedly altered by changes in photoperiod length (Lewis et al., 2009; Schwean-Lardner et al., 2012a). Changes in feeding behavior are anticipated to affect the size of the upper gastrointestinal tract which consistently responds to changes in photoperiod (Savory, 1976). In general, maximum feeding activity in broilers can occur after initiation of photo/light phase or prior to the onset of the dark period (Savory, 1980; Buyse et al., 1993, Duve et al., 2011; Schwean-Lardner et al., 2014). Compared to the morning "hunger" initiated feed intake surge for poultry birds, an increase in feed intake prior to the end of day resulting in a 10 fold increase in the amount of dry matter stored in the crop, has been speculated to reflect a learned strategy to cope with the nightly fast (Buyse et al., 1993, Duve et al., 2011). This extensive and extended storage of feed in the crop might impact the abundance of the primary inhabiting bacteria, lactobacilli (Fuller and Brooker, 1974), which typically act on the stored food to produce several carboxylic acids, which results in a drop in crop pH (Scanes and Pierzchala-Koziec, 2014). Furthermore, due to its anterior most location in chicken GIT, low pH and higher lactobacilli inhabitance, the crop has been suggested to prevent pathogen colonization in the crop and as well as in the entire poultry GIT (Durant et al., 1999; Hinton et al., 2000a; Cutler et al., 2005; Scanes and Pierzchala-Koziec, 2014).

Likewise, considerable investigations has been directed at determining dietary amino acid requirements for growth, feed conversion and breast yield (Hickling et al., 1990; Kidd et al., 2004; Corzo et al., 2005; Brickett et al., 2007). Typically, optimal levels of dietary amino acids are fed to fulfill requirements of each growth stage of broiler chickens (Corzo et al., 2005). For modern high yielding broilers, providing suboptimal levels of dietary amino acid levels have been shown to reduce growth, breast meat yield

and impair bird's immune responses (Corzo et al., 2005; Li et al., 2007). Moreover, it has been suggested that broilers amino acid requirements might change, depending on their growth rate when raised under different photoperiod regeimes. Broiler raised under continuous photoperiod with high growth rate has shown increased feed conversion efficiency with 112% NRC dietary methionine (recommendations) levels versus 16L:8D photoperiod raised counterparts (Hickling et al., 1990).

Based on these findings, it was hypothesized that reduced photoperiod exposure would increase utilization of the crop for feed storage altering the abundance and composition of native microbiota due to changes in substrate availability. Further, the demonstrated effects of photoperiod on bird performance, breast meat yield and immunity suggest that amino acid requirements supporting maximum performance and breast meat yield may be affected by photoperiod. Therefore, the purpose of the current experiment was to examine the effect of photoperiod and dietary amino acid levels collectively on performance, breast meat yield, and microbial ecology, in broiler chickens.

3.3 Materials and Methods

3.3.1 Experimental Design and Bird Management

All experiments using broiler chickens were approved by the University of Saskatchewan Animal Care Committee following the recommendations of the Canadian Council of Animal Care under protocol number 19940248. Day-old Ross X Ross 308 chicks (n=6294) were randomly placed in nine rooms and each room was further divided into twelve pens (2 x 2.3 m). Housing density at the end of the trial did not exceed 30 kg/m^2. Birds were raised to 36 d and had *ad-lib* access to water via Lubing nipple drinkers (Lubing Systems LP, Cleveland, TN, USA; six nipples per pen). Pelleted corn and soybean based diets, including antibiotics and an anticoccidial agent, were formulated on the basis of ideal protein concept, where all other essential amino acids were balanced in proportion to lysine. Diets differ in dietary amino acid content (Low, Medium, High) and were fed on a per bird placed basis; 0.5 kilograms (kg) crumbled starter and 2.0 kg of crumbled grower, and a finisher pellet (4.7 mm diameter) until trial end at 36 d (Table 3.1).

Room temperature was maintained at 34 °C on 0 d and was gradually reduced to 22 °C by 35 d. From placement (0 d) to 7 d of age, all rooms were given 23L with a minimum light intensity of 20 lux. At 7 d, rooms were randomly allocated to selected photoperiod treatments, and adjusted to a minimum light intensity of 7.5 lux. Ventilation was adjusted to match the bird number and bird weight, in each room and ammonia concentrations were not allowed to exceed 25 ppm. Fresh and clean wheat straw was placed within the pens as a bedding material, to a depth of 7.5 to 10 cm prior to placement of the chicks.

Table 3.1. Composition of experimental diets and their calculated nutrient content.

Ingredients, g/kg	Starter			Grower			Finisher		
	Lo[1]	Med	Hi	Lo	Med	Hi	Lo	Med	Hi
Corn	665.00	619.00	580.00	684.00	651.00	601.00	716.00	676.00	630.00
Soybean Meal	243.00	280.00	312.00	216.00	242.00	284.00	175.00	206.00	245.00
Meat & Bone Meal	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
Limestone	10.70	10.60	10.50	8.60	8.50	8.40	8.30	8.20	8.10
Dicalcium Phosphate	8.30	8.10	7.90	7.60	7.40	7.20	7.00	6.80	6.60
Tallow/Vegetable Oil	7.00	16.00	23.00	20.00	26.00	35.00	31.00	39.00	47.00
Broiler Premix[2]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Salt	3.70	3.80	3.80	3.80	3.80	3.80	3.80	3.80	3.80
DL-Methionine	2.05	2.32	2.80	1.23	1.71	1.94	0.92	1.18	1.49
Lysine	1.84	1.90	2.13	0.86	1.28	1.19	0.76	1.00	0.99
Choline Chloride	1.00	1.00	1.00	0.80	0.80	0.80	0.70	0.70	0.70
Monteban[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
BMD[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Threonine	0.17	0.33	0.47	0.00	0.13	0.22	0.00	0.00	0.00
Calculated Nutrients analysis									
Crude Protein,%	21.5	22.5	23.7	20.0	21.0	22.4	18.0	19.2	20.7
ME, kcal/kg	3,000	3,000	3,000	3,100	3,100	3,100	3,200	3,200	3,200
Lysine, %	1.25	1.35	1.45	1.10	1.20	1.30	0.98	1.08	1.18
Arginine, %	1.37	1.48	1.57	1.23	1.34	1.46	1.10	1.21	1.32
Methionine, %	0.55	0.60	0.63	0.47	0.52	0.56	0.42	0.46	0.507
TSAA, %	0.94	1.01	1.08	0.83	0.90	0.98	0.74	0.81	0.88
Threonine, %	0.85	0.92	0.98	0.77	0.84	0.975	0.69	0.76	0.83
Tryptophan, %	0.23	0.25	0.26	0.21	0.23	0.25	0.18	0.21	0.23
Valine, %	0.98	1.04	1.11	0.89	0.97	1.05	0.79	0.87	0.96
Isoleucine, %	0.97	0.98	1.00	0.78	0.85	0.92	0.70	0.85	0.84
Calcium, %	0.97	0.97	0.97	0.85	0.85	0.85	0.82	0.82	0.82
Available Phosphorus, %	0.45	0.45	0.45	0.43	0.43	0.43	0.41	0.41	0.41
Sodium, %	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20

[1]Amino acid level; Lo=Low, Med= Medium, Hi=High.

[2]Supplied per kilogram of diet: vitamin A 11000 IU; vitamin D 2200 IU; vitamin E 30 IU; menadione 2 mg; thiamine 1.5 mg; riboflavin 6 mg; pyridoxine 4 mg; vitamin B12 0.02 mg; niacin 60 mg; pantothenic acid 10 mg; folic acid 0.6 mg; biotin 0.15 mg; copper 10 mg; iron 80 mg; manganese 80 mg; iodine 0.8 mg; zinc 80 mg; selenium 0.3 mg; calcium carbonate 500 mg; antioxidant 0.62 mg.

[3]Active ingredient Narasin (Elanco, Canada Inc.).

[4] Bacitracin Methylene Disalicylate (Zoetis Canada Inc.).

3.3.2 Growth Performance and Meat Yield

Body weight and feed intake per pen was assessed at 7, 29 and 36 d of age. Mortality and culls were collected and evaluated as described by Schwean-Lardner et al. (2013). Birds were checked twice a day by Poultry Centre staff and those demonstrating abnormality (skeletal, behavioral, loss of body weight, illness symptoms) were euthanized by cervical dislocation. Dead and culled birds were further necropsied for cause of death, or morbidity (details in Chapter 2). Meat yield was assessed in 39 d old broiler (30 males and 30 females per diet x photoperiod treatment) by recording feed-restricted (feed (4 h) and water (additional 2 h)) live bird weight, whole carcass weight (excluding neck), and weight of the dissected *pectoralis minor* and *pectoralis major*.

3.3.3 Sample Collection at Euthanasia

One bird was randomly selected from each of 12 pens in each of nine rooms (n=108), weighed and euthanized on 7, 12, 19, 26 and 33 d, by cervical dislocation. Empty crop weight was determined and recorded relative to body weight. The crop pH was determined using a pH meter (Beckmann Φ50 pH meter, CA, USA) after mixing of 1 g content in 9 mL of ddH_2O. The entire content of the crop was mixed, subsamples and snap frozen in liquid nitrogen prior to storage at -80 °C until analysis.

3.3.4 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Crop content from 10 randomly selected birds collected at 33 d of age, representing the two extreme photoperiod treatments (13L and 23L) and three different amino acid levels ($n \geq 5$), were subjected to microbiota analysis (minimum 1 g) by terminal-restriction fragment length polymorphism (T-FRLP) and quantitative polymerase chain reaction (qPCR). Briefly, DNA was extracted from 350 mg of crop contents after some modification of the method described by Dumonceaux et al. (2006). The contents were transferred into Bead beater tubes (Mo-Bio Laboratories, Solano Beach, CA). Bacterial cells were lysed enzymatically and chemically by 365 µL of buffer B1 (containing 0.5 % of Triton X-100 and Tween 80) with RNase A (10 mg/mL), 7.5 µL of lysozyme (100 mg/mL concentration) and 20 µL of proteinase K (20 mg/mL). Tubes were incubated for 1 h at 37 ºC. Samples

were again incubated for 30 min at 50 ºC after mixing with 135 µL of buffer B2 (containing 675 mM of Guanidine-HCl and 4 % of Tween 20). Samples were then frozen at -70 ºC for 30 min. After thawing, 700 µL of pheonl-chloroform-isoamyl alcohol (25:24:1) (Sigma) was added. Samples were shaken three times for 20 s at a speed setting of 5, in a bead beater instrument (Bio 101 ThermoSavant FP120). The top phase was then separated after centrifugation at 14,600 X g for 15 min and mixed with equal volumes of chloroform. The top phase was again removed and mixed with 70 µL volume of 3M sodium acetate and 700 µL of isopropanol. The pellet was then washed (centrifugation 14,600 X g for 15 min) with 70 % ethanol, air dried and dissolved in 100 µL of UV sterile water.

PCR amplification of each sample was carried out using 16S rRNA specific universal primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 926R (5' CCGTCAATTCCTTTRAGTTT 3') (Fernando et al., 2010). The forward primer (8F) was labeled with 6'-carboxyfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide (6-FAM) at the 5' end. The reaction mixture (50 µL) contained 1 µL of the template DNA, 5 µL of 10 X incubation buffer, 1.5 µL of 50 mM $MgCl_2$, 1.3 µL of each primer (10 µM), 1.5 µL of each deoxynucleoside triphosphate (10mM), 0.2 µL of Taq polymerase (Invitrogen) and 38.5 µL of UV-sterilized Millipore water. Amplification was carried out in Ependorf Mastercyler EP (Hamburg, Germany) using the following conditions: 94 ºC for 5 min, 34 cycles of 94 ºC for 40 sec (s), 55 ºC for 40 s, 72 ºC for 1 min and final extension at 72 ºC for 7 min. To check for the desired PCR product size, 5 µL of the PCR products were then analyzed in 1.5 % agarose gel electrophoresis in 1X TAE buffer containing 0.5 µg ethidium bromide/mL of agarose. A QIAquick column purification kit (Qiagen, USA) was used to purify the PCR products following the manufacturer's instructions. The concentration of purified PCR products was measured by absorbance (260 nm) using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and stored at -20 ºC. Purified PCR products were digested for 18 h at 37 ºC in a reaction containing 200 ng of purified PCR product, 20 U of Msp1 restriction enzyme (Fermentas, EU) in 2 µL of reaction buffer and an appropriate amount of UV-sterilized Millipore water, to a total volume of 20 µL. Digested restriction product (2

μL/sample) was mixed with 9 μL of Hi-DiTM formamide (Genetic Analysis Grade, Applied Biosystems) and 0.5 μL of internal LIZ size standard (GenescanTM, Applied Biosystems, USA) and identified by the ABI 3130X capillary sequencer (ABI Applied Biosystems, Foster City, CA, USA). Before placing the samples in the sequencer, each sample was denatured at 95 ºC for 2 min and immediately cooled on ice. T-RFLP profile was imaged using GeneMapper v3.7 software (Applied Biosystems, USA) providing data corresponding to terminal restriction fragment size (base pairs) and peak area, which were estimated in reference to the internal standard. Fragments were differentiated using a binning criteria of ± 3 bp (Metzler-Zebeli et al., 2010). Taxonomic assignments for TRFs were obtained by *in silico* Msp1 and HaeIII digestion of 8F and 926R amplicons using tools available at **http://mica.ibest.uidaho.edu/digest.php** and compared against the Ribosomal Database Project (RDP) database (Metzler-Zebeli et al., 2010).The relative abundance of each TRF was calculated as the peak area of the respective TRF, divided by the total peak area of all TRFs detected within a fragment length range, between 50 and 600 bp (Fernando et al., 2010). Data were manually normalized by removing the TRFs below 1 % of the relative peak area (Fernando et al., 2010). Further, for each sample, two fingerprint replicates of the bacterial community were obtained and bands present in both replicates were subject to analysis using Bionumerics software version 5.1 (Applied Maths, TX, USA). Band based fingerprint, unweighed, pair group method using arithmetic averages for (UPGMA) cluster analysis was performed using the Dice coefficient (band presence/absence, as well as option 1 % position tolerance, also used here). The dendrogram generated represents the grouping and relatedness between samples, whereas the coefficient bar above each diagram illustrates the relative similarity between samples (Teirlynck et al., 2009a).

3.3.5 Quantitative Polymerase Chain Reaction (qPCR)

Selected bacterial taxa and species were enumerated by qPCR using iQTM SYBR Green Supermix (Bio-Rad Laboratories Inc., CA) and a CFX96 Real-Time PCR Detection System with a C1000 thermal cycler (BioRad, Guénette, Canada). The 25 μL reaction mix contained 12.5 μL Platinum SYBRGreen qPCR Supermix UDG (Invitrogen, Carlsbad, CA,

USA), 0.8 µM of each primer, calibration standards or 2 µL of template DNA mixed in 6.5 µL of double distilled sterilized water. Briefly, the amplification program for any bacterial target consists of following steps: 95 ºC for 3 min followed by 40 cycles of 95 ºC for 40 s, bacterial target specific annealing temperature and primers (Table 3.2) for 40 s and extension at 72 ºC for 40 s. Fluorescence data was collected at the annealing or annealing/extension step. *L. gallinarum* PCR primers were designed during this study by using cpn-60 UT sequence (ATCC 33199 from http://www.ncbi.nlm.nih.gov/nuccore/NR_117061.1) in Beacon Designer™ software (Premier BioSoft International, Palo Alto, CA, USA). The copy number was calibrated to standard curve (10-fold dilution series) generated using pooled PCR product containing the DNA sequence corresponding to the target bacteria gene (Wise and Siragusa, 2007). Standard curves for each crop bacterial target qPCR assay were generated using gel purified (QIAEX II, Qiagen) amplicons using a method described by Fernando et al. (2010).

Table 3.2. Primers used for detection and enumeration of selected major bacterial groups and species by PCR and qPCR in this study.

Bacterial Target	Amplicon size (bp)	Annealing temp. (°C)	Sequence (5'-3')	Reference
Total bacteria[1] (V3 region)	200	60	F-CGGYCCAGACTCCTACGGG R-TTACCGCGGCTGCTGGCAC	Lee et al., 1996
Streptococcus spp[1].	485	57	F-GTTAGCCGTCCCTTTCTGG R-GAGTTTGATCCTGGCTCAG	Franks et al.,1998
Clostridium leptum subgroup [1]	239	50	F-GCACAAGCAGTGGAGT R-CTTCCTCCGTTTTGTCAA	Matsuki et al., 2004
Bifidobacterium spp[1].	550-563	56	F-CTCCTGGAAACGGGTGG R-GGTGTTCTTCCCGATATCTACA	Matsuki et al., 2002
Enterococcus[1]	144	61	F-CCCTTTATTGTTAGTTGCCATATT R-ACTCGTTGTACTTCCCATTGT	Rinttila et al., 2004
Enterococcus fecalis (cpn-60)[2]	100	55	F-AAGTTGGTAACGACGGCGTAA R-GATAAATAACCGCGGTCGAAT	Dumonceaux et al., 2006
Total *Lactobacillus spp.*[1]	346	55	F-GCAGCAGTAGGGAATCTTCCA R-GCATTYCACCGCTACACATG	Walter et al., 2001
Lactobacillus crispatus (cpn-60)[2]	125	59	F-TCCTTACATTTTGATCACTGA R-GAGCTTCACCAGTAACGTC	Dumonceaux et al., 2006
Lactobacillus johnsonii (cpn-60)[2]	146	55	F-TACTATTGAAGAATCAAAGGG R-TCAGTAATCAAAATGTAAGGG	Dumonceaux et al., 2006
Lactobacillus salivarius[1]	332	60	F- CGAAACTTTCTTACACCGAATGC R- GTCCATTGTGGAAGATTCCC	Feng et al., 2010
Lactobacillus reutri[1]	305	58	F-CAGACAATCTTTGATTGTTTAG R-GCTTGTTGGTTTGGGCTCTTC	Feng et al., 2010
Lactobacillus gallinarum[3]	167	58	F- GCTAACCCAGTTGGCATTCG R-TCCATAGCGTCAGCAATCAAAG	Designed in this study

[1]16S rRNA gene based primers.
[2]cpn-60 gene based primers.
[3]Primers were designed during this study, by using cpn-60 Universal Target sequence (ATCC 33199) from http://www.ncbi.nlm.nih.gov/nuccore/NR_117061.1 in Becan Designer ™ software (PREMIER Biosoft, USA)

3.3.6 Statistical Analyses

Data collected prior to initiation of treatment photoperiod programs at 7 d of age were omitted for statistical analysis. The performance, breast yield, mortality and crop data were analyzed using PROC GLM of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA) using a 3x3x2 factorial arrangement where rooms were nested within photoperiod programs. The model included the main effects of photoperiod (3), amino acid level (3), and gender (2), as well as 2- and 3-way interactions that could have occurred between these factors. For performance, breast meat yield and mortality data, pen (n=54) was regarded as replicate unit when analyzed for dietary amino acid levels and gender whereas room was replicate unit for photoperiod, however, for crop parameters, individual bird constituted the replication unit. Relative crop weight (g organ weight/g bwt) was log transformed prior to statistical analysis. For regression, PROC REG and PROC RSReg in SAS (9.1) was used to analyze the relationship crop parameters and photoperiod or dietary amino acid levels. Differences were considered significant when the probability was less than, or equal to, 0.05. Shannon-wiener, Evenness and Simpson diversity indices for total microbial community composition was calculated based on the relative peak areas for Msp1 and Hae III digested T-RFLP profiles as ecological measures of the relative distribution of microbial groups in the community (Metzler-Zebeli et al., 2010). Crop content diversity indices and qPCR analysis conducted for selected bacterial target enumeration, were statistically assessed using a 3x3 factorial arrangement where photoperiod, amino acid level and their interaction were included in the model by Proc Mixed in SAS (9.1) and individual birds as the replicate unit. The correlation between body weight and the relative abundance of 177 bp TRF, recorded after Msp1 digestion of crop contents, were evaluated by using the Spearman coefficient in SAS (9.1).

3.4 Results

3.4.1 Growth Performance and Feed Efficiency

Performance measurements are summarized in Table 3.3. Body weight and average gain were reduced under 13L compared with 18L and 23L, which were similar. Feed

consumption increased with each increase in photoperiod length. Feed efficiency (7-36 d) and mortality corrected feed efficiency (29-36 d and 7-36 d) was higher for 13L exposed broilers compared to 18L and 23L exposed birds both having similar values. The Lo amino acid level reduced body weight at 29 and 36 d compared to the Med and Hi treatments. The impact of amino acid level was the result of differences in gain between 7 and 29 d as dietary treatment did not affect gain between 29 and 36 d. From 7 to 29 d and 7 to 36 d, feed efficiency (G:F) was higher for the Med and Hi treatments that the Lo treatment. This parameter was unaffected by dietary amino acid level from 29 to 36 d of age. Mortality corrected G:F increased with each increase in dietary amino acid level from 7 to 29 and 7 to 36 d of age, whereas the Lo treatment value was lower than the Med and Hi treatments (not different) for the 29 to 36 d period. As expected, male broilers consistently outperformed females with increased body weight, average weight gain, and improved feed efficiency.

Only a few significant interactions were observed for performance data. In contrast to our hypothesis, no interaction between photoperiod and amino acid level was observed. Photoperiod by gender effects were observed (Table 3.4) such that body weight gain (29-36 d) decreased with increasing daylength for males, but the effect for females did not follow an obvious trend. Feed efficiency during the finishing period (29-36 d) decreased with increasing daylength for males whereas the value for females was highest for the 13L birds and similar for the 18L and 23L treatments. For females from 7 to 29 d, mortality corrected feed efficiency decreased with increasing daylength, whereas for males the 13L and 23L values were similar and higher than the 18L value.

A significant gender by amino acid level interaction was observed for mortality corrected feed efficiency examined from 7-36 d (Table 3.5) period. For both genders, mortality corrected feed efficiency increased with level of dietary amino acids, but in females the Med value was more similar to the Hi value than what was found for males.

Table 3.3. Effect of photoperiod, amino acid level and gender on performance parameters of broiler chickens (n=54).

Para-meter	Photoperiod (PD)[1]			Amino Acid Level[2] (AA)			Gender (GD)[3]		P-value						SEM
	13L	18L	23L	Lo	Med	Hi	M	F	PD	AA	GD	PDx AA	PDx GD	AAx GD	
Body weight (kg)															
7 d	0.16	0.16	0.17	0.16^{b}	0.17^{a}	0.17^{a}	0.17	0.16	NS	0.005	NS	NS[4]	NS	NS	0.001
29 d	1.38^{b}	1.49^{a}	1.52^{a}	1.43^{b}	1.48^{a}	1.48^{a}	1.54^{a}	1.39^{b}	0.001	0.001	0.001	NS	NS	NS	0.014
36 d	2.07^{b}	2.17^{a}	2.21^{a}	2.11^{b}	2.17^{a}	2.17^{a}	2.27^{a}	2.03^{b}	0.002	0.001	0.001	NS	NS	NS	0.020
Average gain (kg)															
7-29 d	1.22^{b}	1.32^{a}	1.35^{a}	1.27^{b}	1.31^{a}	1.32^{a}	1.37^{a}	1.23^{b}	0.001	0.001	0.001	NS	NS	NS	0.014
29-36 d	0.69	0.68	0.69	0.68	0.69	0.69	0.74^{a}	0.64^{b}	NS	NS	0.001	NS	0.04	NS	0.008
7-36 d	1.91^{b}	2.00^{a}	2.04^{a}	1.94^{b}	2.00^{a}	2.01^{a}	2.11^{a}	1.86^{b}	0.001	0.001	0.001	NS	NS	NS	0.020
Feed consumed (kg/bird)															
7-29 d	1.85^{c}	2.06^{b}	2.13^{a}	2.03	2.02	2.00	2.10^{a}	1.93^{b}	0.001	NS	0.001	NS	NS	NS	0.021
29-36 d	1.15^{b}	1.18^{ba}	1.21^{a}	1.18	1.19	1.17	1.24^{a}	1.12^{b}	0.020	NS	0.001	NS	NS	NS	0.011
7-36 d	3.02^{c}	3.25^{b}	3.36^{a}	3.22	3.21	3.20	3.37^{a}	3.05^{b}	0.001	NS	0.001	NS	NS	NS	0.031
Feed efficiency (G:F[4]) (g/g)															
7-29 d	0.65	0.64	0.63	0.62^{c}	0.65^{b}	0.66^{a}	0.65^{a}	0.64^{b}	NS	0.001	0.005	NS	NS	NS	0.003
29-36 d	0.59^{a}	0.57^{ab}	0.56^{b}	0.56	0.58	0.57	0.58^{a}	0.56^{b}	0.033	NS	0.020	NS	0.05	NS	0.004
7-36 d	0.63^{a}	0.61^{b}	0.60^{b}	0.60^{b}	0.62^{a}	0.63^{a}	0.62^{a}	0.61^{b}	0.001	0.001	0.001	NS	NS	NS	0.003
Feed efficiency (g/g) with mortality corrected (G:F^{m}[5])															
7-29 d	0.66	0.65	0.65	0.64^{c}	0.66^{b}	0.67^{a}	0.67^{a}	0.64^{b}	0.056	0.001	0.001	NS	0.03	NS	0.003
29-36 d	0.60^{a}	0.57^{b}	0.57^{b}	0.57^{b}	0.58^{a}	0.59^{a}	0.60^{a}	0.57^{b}	0.003	0.002	0.001	NS	NS	NS	0.004
7-36 d	0.65^{a}	0.63^{b}	0.63^{b}	0.62^{c}	0.64^{b}	0.65^{a}	0.65^{a}	0.63^{b}	0.004	0.001	0.001	NS	NS	0.03	0.003

SEM-Standard error of the mean.

[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D; [2]AA; Lo=Low, Med= Medium, Hi=High; [3]GD; M=Male, F=Female.

[4]G:F=(final period weight -initial period weight)/period feed consumption.

[5]G:F^{m}=(final period weight + kg of mortality weight-initial period weight)/period feed consumption.

[a–c] Means within a row and main effect with no common superscript differ significantly ($P < 0.05$).

Table 3.4. Interaction effect between photoperiod and gender for average gain, gain to feed (G:F)[1] and gain to feed mortality corrected (G:F^m)[2] (n=54).

Period	Gender[3]	Photoperiod[4]		
		13L	18L	23L
Average gain (kg/g)				
29-36 d	M	0.749	0.739	0.722
29-36 d	F	0.632	0.621	0.654
Feed efficiency (g/g)				
29-36 d	M	0.595	0.586	0.556
29-36 d	F	0.585	0.549	0.557
Feed efficiency mortality corrected (g/g)				
7-29 d	M	0.674	0.662	0.671
7-29 d	F	0.655	0.642	0.637

[1]G:F=(final period weight - initial period weight)/period feed consumption.
[2]G:F^m=(final period weight + kg of mortality weight-initial period weight)/period feed consumption.
[3]Gender; M=Male, F=Female.
[4] Photoperiod; 13L=13L:11D, 18L=18L:6D,23L=23L:1D.

Table 3.5. Interaction effect of amino acid level and gender on mortality corrected gain to feed (G:F^m)[1] ratio (g/g) (n=54).

Period	Gender[2]	Amino Acid Level [3]		
		Lo	Med	Hi
7-36 d	M	0.625	0.645	0.657
7-36 d	F	0.605	0.620	0.625

[1]G:F^m=(final period weight + kg of mortality weight-initial period weight)/period feed consumption.
[2]Gender; M=Male, F=Female.
[3]Amino acid level; Lo=Low, Med= Medium, Hi=High.

3.4.2 Breast Meat Yield

The main effects of photoperiod, amino acid level and gender on 39 d old broiler chicken breast meat yield, proportional to live weight, are shown in Table 3.6. Carcass yield was lower for the 13L than 18L and 23 L treatments. Increasing photoperiod length increased *Pectoralis major*, *Pectoralis minor* and total breast meat breast yield with each daylength statistically independent of one another. Diet amino acid level did not affect cacass yield, but feeding the Lo amino acid level led to a decrease in breast meat yield, compared to the Med and Hi levels, which were similar in amount. Gender did not affect carcass yield, but females had larger proportional *Pectoralis minor* and total breast weights. Interaction effects between photoperiod and amino acid level, were detected only for carcass weight (calculated percent of live weight) (Table 3.7). Increasing photoperiod length and supplementing with higher amino acid levels resulted in heavier broiler carcasses while broiler provided with 13L and high amino acid demonstrated lowest carcass yield.

Table 3.6. Effect of photoperiod, amino acid level and gender on breast meat yield (% of live weight) of 39 d old broiler chickens (n=540).

Item	Photoperiod (PD)[1]			Amino Acid Level (AA)[2]			Gender (GD)[3]		P-value						SEM
	13L	18L	23L	Lo	Med	Hi	M	F	PD	AA	GD	PD x AA	PD x GD	AA xGD	
Carcass	66.86^{b}	68.25^{a}	68.41^{a}	67.88	68.06	67.58	67.93	67.75	0.01	NS	NS	0.001	NS	NS	0.095
Pectoralis major	14.39^{c}	15.34^{b}	15.91^{a}	14.86^{b}	15.32^{a}	15.43^{a}	15.12	15.29	0.01	0.001	NS	NS	NS	NS	0.062
Pectoralis minor	3.42^{c}	3.54^{b}	3.66^{a}	3.46^{b}	3.53^{b}	3.63^{a}	3.39^{b}	3.69^{a}	0.01	0.001	0.001	NS	NS	NS	0.020
Total breast	17.81^{c}	18.88^{b}	19.57^{a}	18.32^{b}	18.85^{a}	19.06^{a}	18.52^{b}	18.98^{a}	0.01	0.001	0.002	NS	NS	NS	0.071

SEM-Standard error of the mean.

[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D.

[2]AA; Lo=Low, Med= Medium, Hi=High.

[3]GD; M=Male, F=Female.

[a–c]Means within a row and main effect with no common superscript differ significantly ($P < 0.05$).

Table 3.7. Interaction effect of photoperiod and amino acid level on the proportionate carcass yield (% of live weight) of 39 d old broiler chicken (with bird as replicate unit, n=540).

	Photoperiod (PD)[1]		
AA[2]	13L	18L	23L
Lo	67.00	68.37	68.32
Med	67.79	68.19	68.20
Hi	65.80	68.19	68.70

[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D.
[2]AA; Lo=Low, Med= Medium, Hi=High.

3.4.3 Crop

Relative crop weight and crop pH values are shown in Table 3.8. The empty crop relative weight from birds exposed to the shorter photoperiod (13L) was significantly higher ($P \leq 0.002$) in comparison to longer photoperiods (18L and 23L) at all time points of this study (12, 19, 26 and 33 d). It should be noted that the value for the 18L treatment was intermediate in value, and was not statistically different from the 23L treatment. Photoperiod also affected crop pH after 19 d of age, with values lower for shorter daylengths (linear – 26 and 33 d; quadratic – 19 d). Amino acid level did not affect crop size and pH except at 33 d crop pH increased with increasing diet amino acid level. Gender did not affect crop size or pH and no interactions were found between main effects for these traits.

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Table 3.8. Effect of photoperiod, amino acid level and gender on the crop size and pH in broilers (n=108).

Crop	Age (d)	Photoperiod (PD)[1]			Amino Acid Level (AA)[2]			Gender (GD)[3]		P-value						SEM
		13L	18L	23L	Lo	Med	Hi	M	F	PD	AA	GD	PDx AA	PDx GD	AAx GD	
Relative Size (% mg/g bwt)																
	12	2.59[a]	1.26[b]	0.74[b]	1.56	1.53	1.51	1.59	1.47	0.0003	NS	NS	NS	NS	NS	0.104
	19	1.73[a]	0.69[b]	0.49[b]	1.00	0.91	1.01	0.98	0.96	<.0001	NS	NS	NS	NS	NS	0.074
	26	1.51[a]	0.77[b]	0.46[b]	0.86	0.92	0.98	0.99	0.84	0.0006	NS	NS	NS	NS	NS	0.067
	33	1.93[a]	0.99[b]	0.73[b]	1.22	1.15	1.28	1.26	1.18	0.002	NS	NS	NS	NS	NS	0.085
pH																
	12	5.03	5.28	5.13	5.26	5.05	5.13	5.16	5.13	NS	NS	NS	0.03	NS	NS	0.055
	19*	5.14	5.51	5.28	5.34	5.21	5.35	5.29	5.30	NS	NS	NS	NS	NS	NS	0.054
	26*	4.74[b]	5.08[a]	5.25[a]	5.02	5.09	4.92	4.99	5.01	0.0031	NS	NS	NS	NS	NS	0.052
	33*	4.76	4.94	5.07	4.83[b]	4.87[ab]	5.08[a]	4.88	4.96	NS	0.04	NS	NS	NS	NS	0.047

SEM-Standard error of the mean.

[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D.

[2]AA; Lo=Low, Med= Medium, Hi=High.

[3]GD; M=Male, F=Female.

[a,b]Means with different superscript within photoperiod, amino acid level and gender are significantly different (P<0.05).

*Significant linear regression of crop pH at 26 and 33 d while quadratic at 19d.

3.4.4 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

A significant level of polymorphism was observed upon digestion with Msp1 for 16S rRNA gene based T-RFLP on crop content samples belonging to the distinct photoperiod regimes, 13L and 23L. Over twenty bands were detected after Msp1 digestion, however, six predominant (> 5 % - 30 % of the mean relative abundance) TRFs of 55, 177-179, 181, 488, 566 and 572-573 bp were observed in more than 60 % of the crop samples (Figure 3.1). Likewise, ten predominant bands (> 5 % - 30 % of mean relative abundance) were observed for crop samples after HaeIII digestion. The TRFs observed were then assigned to bacterial groups after *in-silico* digestion with both Msp1 (Table 3.9) and HaeIII (data not shown) using the online database for bacterial 16S rRNA gene sequences (http://mica.ibest.uidaho.edu/digest.php). The relative abundance of 181 and 572-573 bp TRF, representing primarily *Lactobacillus* spp., was significantly higher in broilers exposed to the shortest photoperiod, i.e, 13L versus 23L (Figure 3.1). For HaeIII digested samples, the 275 bp TRF mean relative abundance, representing mostly uncultured bacteria (Figure 3.1), was found to be highest in the birds exposed to the 23L photoperiod compared to 13L raised birds. Here, the figure 3.2, indicates observed TRFs of 13L crop sample and tentative bacterial species assignment after computer–simulated online *in-silico* digestion of bacterial 16S rRNA library sequence data.

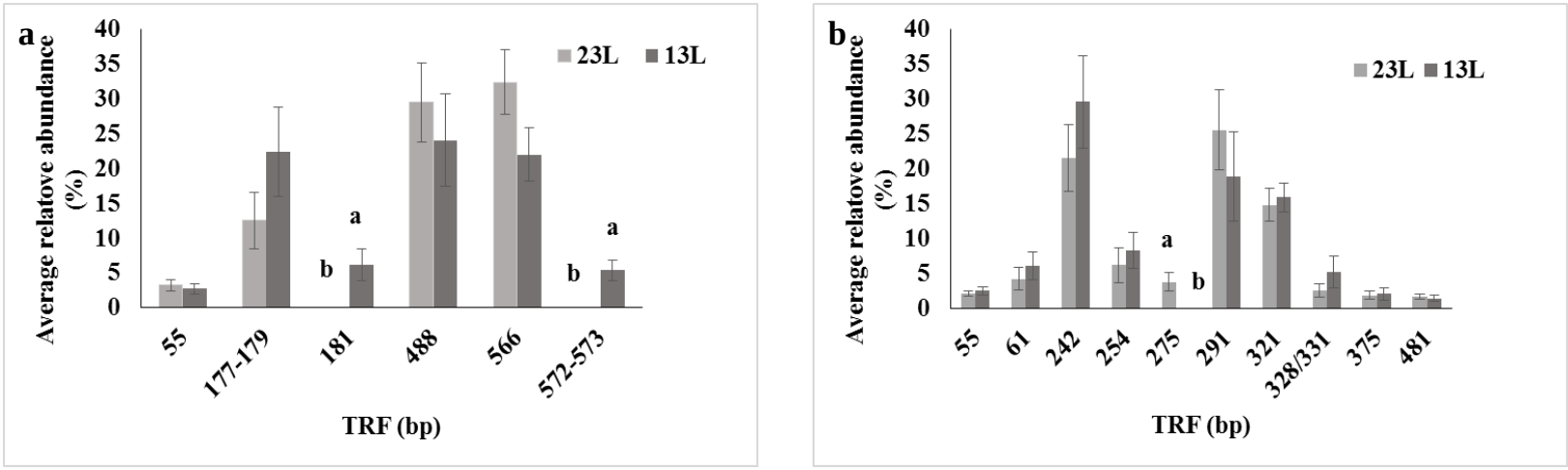


Figure 3.1. Average relative abundance (percent) of prominent TRFs recorded after a) Msp1 and b) HaeIII digestion of crop DNA samples extracted from broiler chickens housed either under 13L:11D or 23L:1D. Vertical lines on bars indicate standard error. Bars with different letters indicate significant ($P<0.05$) difference between photoperiod programs.

Table 3.9. Observed and predicted terminal restriction fragments (TRFs) with Msp1 restriction enzyme for bacterial species and taxonomic assignments by *in-silico* digestion of bacterial 16S rRNA gene sequence data.

Observed size (bp)	Predicted size (bp)	Predicted Bacterial species
55	55-57	Uncultured/unassigned bacteria spp.
177	177-179	Uncultured bacteria, *L. amylovorus, L. delbrukeii, L. helveticus, Lactobacilli spp*.
184	181	*L. helveticus, L. acidophilus, L. amylovorus, L. gallinarum, L. crispatus*
489	488-489	Uncultured rumen bacteria
565	566	*Enterococcus faecalis*
574	572-573	*L. brevis, L. casei, L. plantarum, L. rhamnosus*

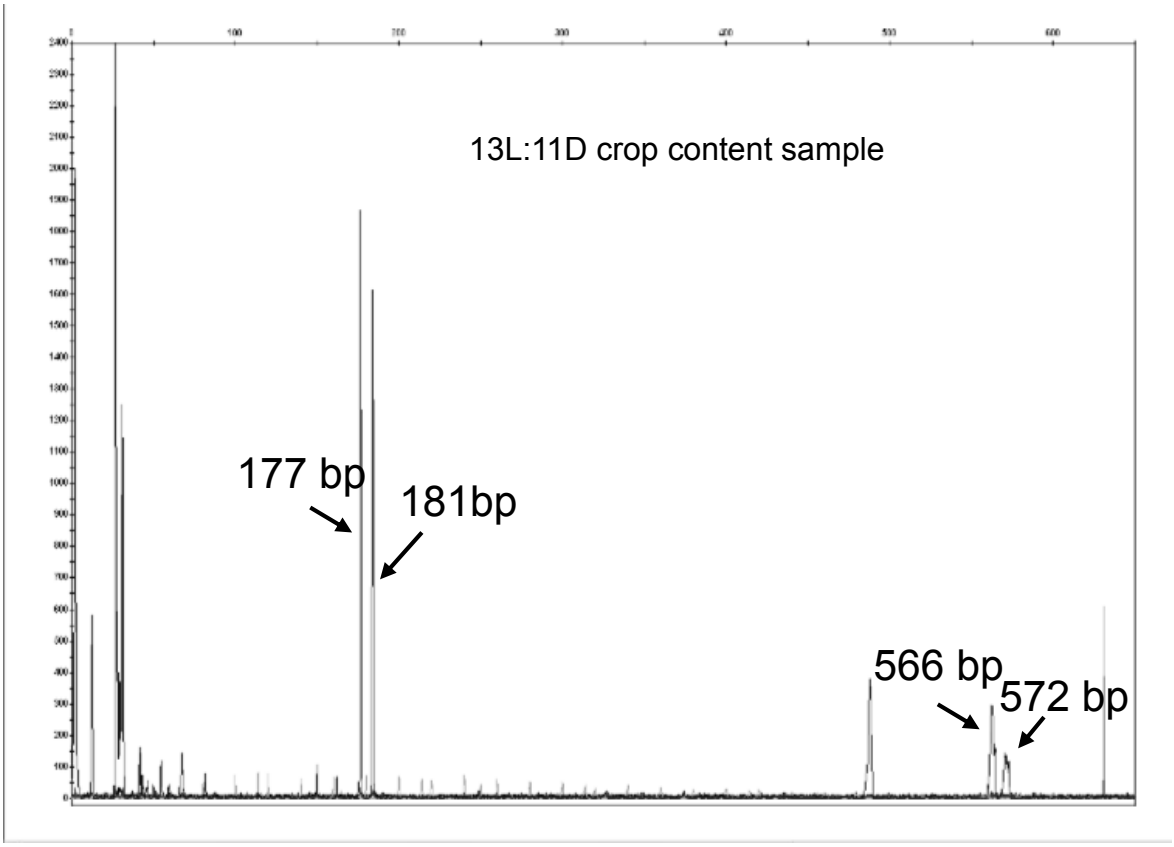


Figure 3.2. T-RFLP analysis of 16S rRNA gene amplified by universal primer (8F and 926R) from bacteria in the crop content of broilers exposed to 13L:11D. Observed TRFs are indicated by arrows which can be assigned to known bacterial targets with computer-simulated online *in-silico* digestion of bacterial 16S rRNA gene sequence data.

3.4.4.1 Cluster, Diversity Indices and Correlation Analysis of T-RFLP data

Analysis of the crop contents for T-RFLP banding patterns by Bionumerics software demonstrated a clustering pattern by photoperiod regimes and amino acid level. The clustering results were identical for Msp1 (Figure 3.3), and HaeIII digested samples (Figure 3.4). None of the crop content diversity indices analyzed here (Evenness, Shannon and Simpson) were significantly affected by the main factors of photoperiod and amino acid level (Table 3.10). Furthermore, the correlation analysis between the body weight of 33 d old broilers and the relative abundance of 177 bp TRF revealed a negative relationship between these two variables (Figure 3.5).

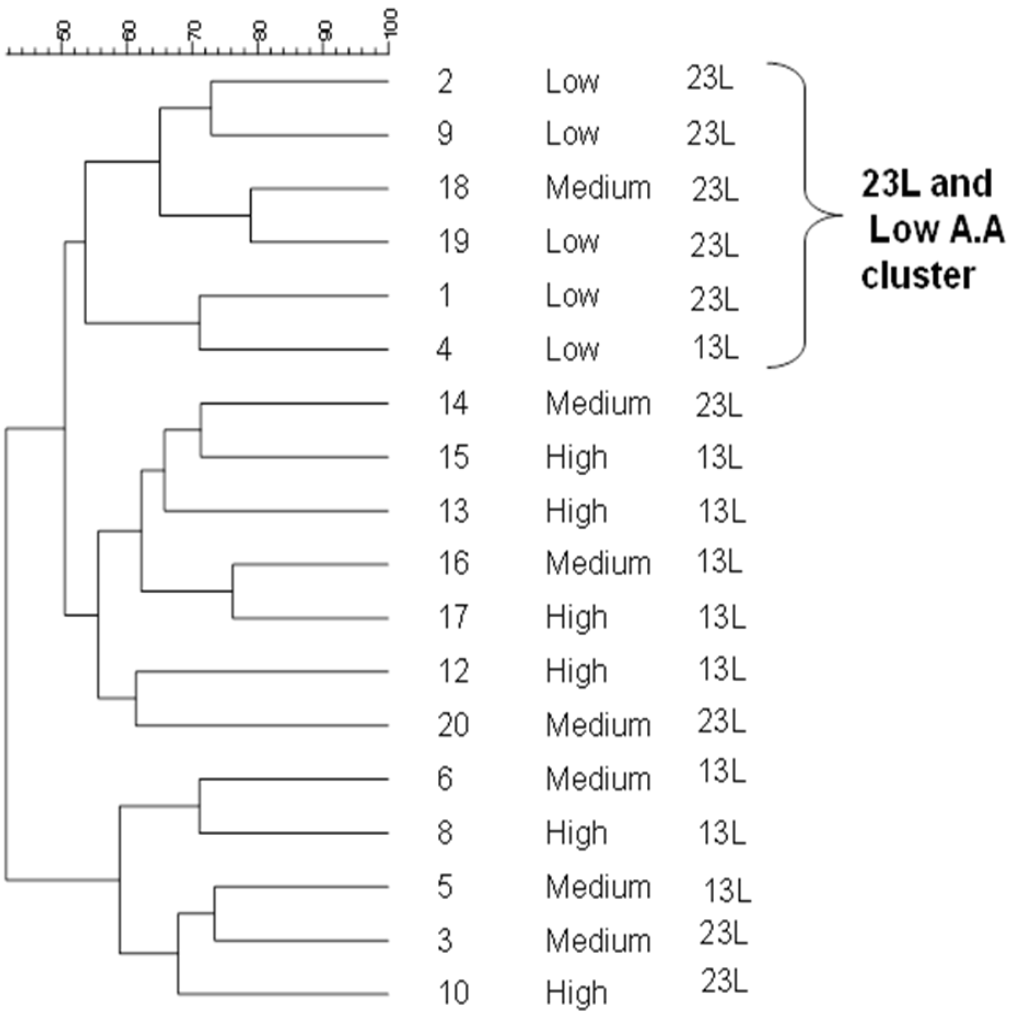


Figure 3.3. Dendrogram showing the relatedness of individual 33 d old broiler, crop microbial community samples, raised under either of two photoperiod lengths (13L or 23L) and fed three levels of amino acid levels (low, medium, high). The relationship was calculated on 16S rRNA based terminal-restriction fragment length polymorphism patterns recorded after Msp1 digestion of individual broiler crop DNA contents. The comparisons were based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. The scale bar above, shows percent relatedness between T-RFLP samples.

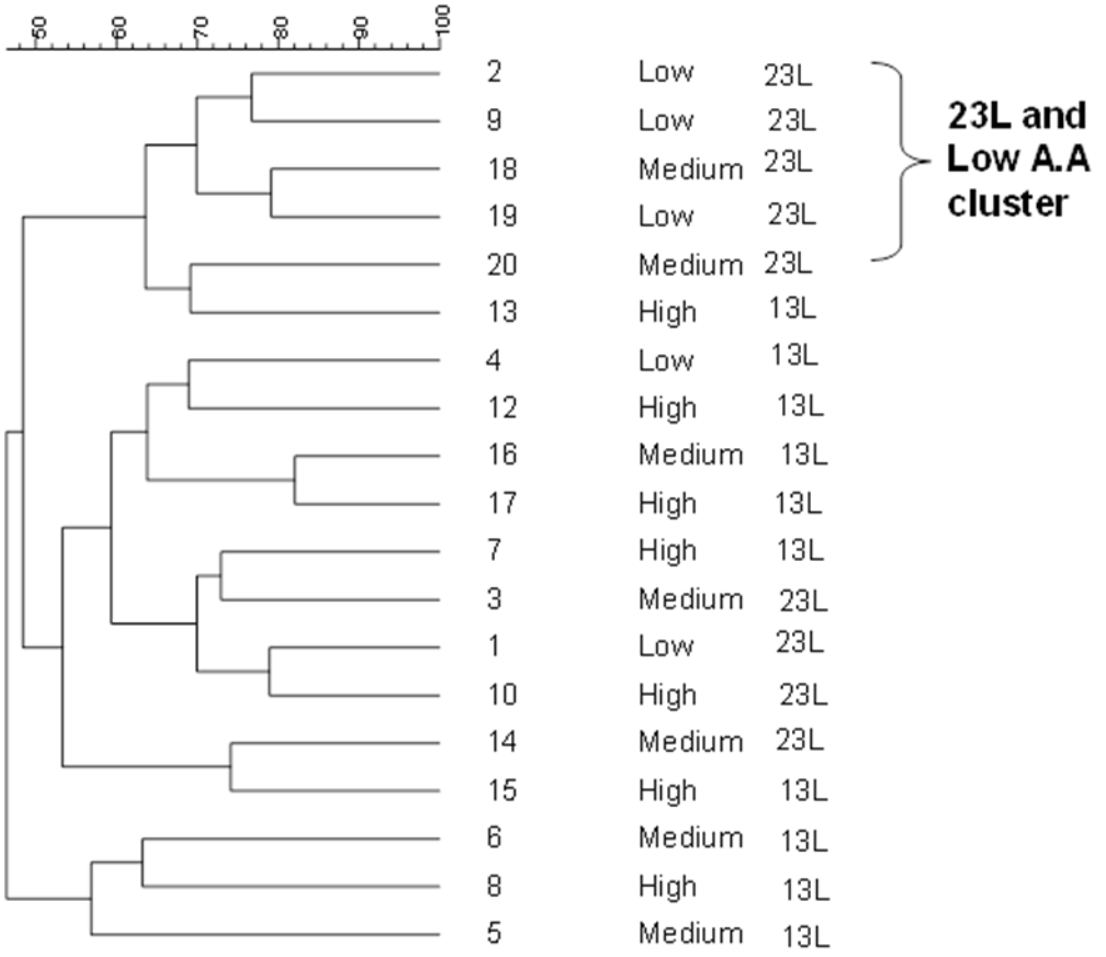


Figure 3.4. Dendrogram showing the relatedness of individual 33 d old broiler crop microbial community samples raised under either of two photoperiod lengths (13L or 23L) and fed three levels of amino acid levels (low, medium, high). The relationship was calculated on 16S rRNA based terminal-restriction fragment length polymorphism patterns recorded after HaeIII digestion of individual broiler chicken crop DNA contents. The comparisons were based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. The scale bar above, shows percent relatedness between T-RFLP samples.

Table 3.10. Effect of photoperiod and amino acid level on crop content microbial community diversity indices generated after restriction enzyme digestion in 33 d old broilers (n=20).

Enzyme	Indices	Photoperiod (PD)[1]		Amino acid Level (AA)[2]			P-value	SEM
		13L	23L	Lo	Med	Hi	PDxAA	
Msp1	Evenness	0.88	0.79	0.88	0.82	0.81	NS	0.020
	Shannon	0.72	0.69	0.73	0.66	0.72	NS	0.027
	Simpson	0.74	0.71	0.75	0.69	0.73	NS	0.017
HaeIII	Evenness	0.78	0.82	0.79	0.78	0.84	NS	0.020
	Shannon	0.75	0.80	0.77	0.74	0.81	NS	0.024
	Simpson	0.74	0.78	0.78	0.73	0.78	NS	0.019

SEM-Standard error of the mean.
[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D.
[2]AA; Lo=Low, Med= Medium, Hi=High.

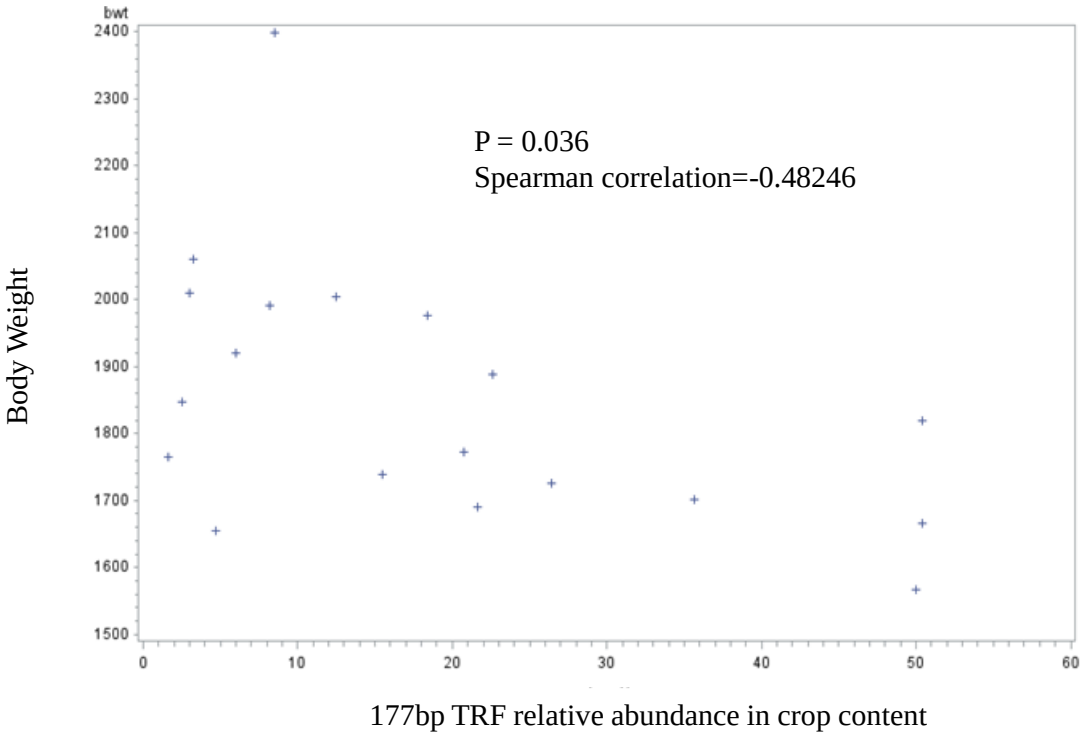


Figure 3.5. The correlation between body weight (g) and relative abundance of 177 bp TRF in the crop contents of 33 d old broilers.

3.4.5 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative enumeration of the major bacterial groups and their member species is shown in Table 3.11. As there were no interactions observed, only the main effects of photoperiod and amino acid level in diet for enumerated bacterial targets are discussed. Total bacterial (P=0.053) and lactobacilli group (P=0.07) count analysis were not significantly different by treatment, however, a trend was revealed, indicating higher counts of these targets in birds reared under 13L versus 23L lit counterparts. The enumeration of selected prominent *Lactobacilli spp*. from the crop, (particularly *Lactobacillus salivarius, L. johnsonii, L. crispatus* and *L. reutri*) also revealed no influence of photoperiod or amino acid level. The only exception was *L. gallinarum*, where counts were significantly reduced in the crop contents of birds reared under 23L (average of 4.72

$\log_{10}$ rRNA gene copy number/g of content) and significantly higher (P=0.0010) under 13L (average of 7.38 $\log_{10}$ rRNA gene copy number/g of content). All other enumerated bacterial targets like *Clostridium leptum subgroup*, *Streptococcus* group, *Enterococcu*s and *Streptococcus agalacticus* were not significantly influenced by either of the two main factors.

Table 3.11. Effect of photoperiod and amino acid level on mean copy number (log_{10} copies of target gene/g of crop content) of selected predominant bacterial groups and species in 33 d old broilers (n=20).

Target Bacteria	Photoperiod (PD)[1]		Amino acid Level (AA)[2]			P-value			SEM
	13L	23L	Lo	Med	Hi	PD	AA	PDXAAa	
Total Bacteria	9.73	9.39	9.66	9.49	9.53	0.053	NS	NS	0.072
Streptococcus group	6.57	5.22	3.78	6.48	7.43	NS	NS	NS	0.707
Clostridium leptum subgroup	6.88	6.54	7.50	6.39	6.24	NS	NS	NS	0.514
Enterococcus group	6.67	5.01	6.10	5.64	5.76	NS	NS	NS	0.451
Streptococcus agalacticus	5.23	5.05	5.38	5.01	5.04	NS	NS	NS	0.095
Lactobacilli group	8.90	7.52	9.06	7.91	7.65	0.07	NS	NS	0.304
*L. gallinarum**	7.38[a]	4.72[b]	6.36	5.93	5.87	0.001	NS	NS	0.367
L. johnsonii	8.15	6.42	8.02	6.71	7.13	NS	NS	NS	0.419
L. crispatus	8.48	8.31	8.12	8.34	8.72	NS	NS	NS	0.119
L. reutri	7.64	7.05	7.39	7.18	7.47	NS	NS	NS	0.161
L. salivarius	7.55	7.88	8.17	7.53	7.45	NS	NS	NS	0.191

SEM-Standard error of the mean.

[1]PD; 13L=13L:11D;18L=18L:6D, 23L=23L:1D.

[2]AA; Lo=Low, Med= Medium, Hi=High.

NS= Not significant (P<0.10).

* Unique observation, 4 out of 10 samples 23L samples failed to generate a reading.

3.5 Discussion

Photoperiod is one of the most commonly implemented management tools used to manipulate the performance (Hassanzadeh et al., 2012; Abbas 2013; Schwean-Lardner et al., 2012a, 2016) and livability of broiler chickens (Olanrewaju et al., 2006; Schwean-Lardner et al., 2013, 2016; Mlaba et al., 2015). Besides growth and livability, several behavior and physiological host processes can also be affected by photoperiod (Olanrewaju et al., 2006, 2015; Schwean-Lardner et al., 2016). Analogous to photoperiod, nutritional alterations, especially dietary amino acid levels, have important effects on growth performance, breast tissue yield (Hickling et al., 1990; Mlaba et al., 2015), feed intake (Zhai et al., 2013) and livability (Lewis et al., 2009) of broiler chickens. However, once popular, and falsely anticipated theory is that a near continuous photoperiod (23L:1D/23L) maximizes the growth rate of modern broilers compared to photoperiod regimes providing more darkness but this is no longer considered to be true (Lewis et al., 2009; Hassanzadeh et al., 2012; Yang et al., 2015b; Schwean-Lardner et al., 2016). Most broiler genetic lines are intensively selected for an accelerated growth pattern, achieving target market weights in short time frames. This accelerated growth might predispose birds to several circulatory, skeletal and metabolic disorders (Olanrewaju et al., 2006; Yang et al., 2015b; Schwean-Lardner et al., 2016). Providing exposure to darkness each day, has been proposed to decrease the negative effects of constant illumination on the immune status (Abbas et al., 2008, Abbas 2013), livability and welfare (Schwean-Lardner et al., 2013). Moreover, previous work from our group has been suggested that a minimum of 4 h to 7 h of darkness exposure per day might be sufficient for typical broiler production, depending on the broiler growth stage and performance targets (Schwean-Lardner et al., 2012a, 2016). Furthermore, compared to 23L, the use of equally performing photoperiod regimes, such as intermittent (4L:4D) or photoperiod restricted (17L:7D), also reduces electricity expenses (Schwean-Lardner et al., 2013; Olanrewaju et al., 2015; Yang et al., 2015b).

Photoperiod duration could also impact broiler chicken feeding behavior, promote feed intake prior to the onset of the dark, depending on photoperiod duration, which in turn may affect crop size (Buyse et al., 1993; Lewis et al., 2009; Duve et al., 2011; Schwean-

Lardner et al., 2012a,b, 2014). Birds typically eat to meet their body energy and growth requirement (Corzo et al., 2005; Dozier et al., 2009), high growing birds might have higher demand for nutrients (energy, amino acids). There has been indication in the past, providing higher methionine (112% NRC) levels to rapidly growing broiler chickens exposed to 23L have shown positive effect on feed conversion compared to 16L:8D group (Hickling et al., 1990). Therefore, it can be speculated that providing higher amino acids to short photoperiod birds might improve broiler performance.

The crop has also been identified as a source of food borne pathogens (*Salmonella enteritidis* and *Campylobacter*) for humans and chickens as a result of entry via the oral route (Scanes and Pierzchala-Koziec, 2014). The pathogen colonization in poultry GIT can be mainly affected by change in the crop microenvironment such as pH and abundance of native microbiota (Durant et al., 1999; Hinton et al., 2000a; Ricke, 2003). In addition, as a result crop and its environment changes, the degree of distal digestive tract colonization and associated organ (spleen, liver) can be directly affected (Durant et al., 1999; Hinton et al., 2000a). However, despite the crop being recognized as critical for prevention of GIT pathogen establishment and gut health, it has received relatively little research attention (Classen et al., 2016). Therefore, the present study was undertaken to concurrently examine effects of photoperiod and amino acid level on comprehensive growth parameters, breast meat yield, crop size and crop microbiota assessment by 16S rRNA gene based molecular profiling techniques.

The results from the current study demonstrated the photoperiod effect on growth parameters was independent of dietary amino acid levels, and similar to previous broiler experiments (Renden et al., 1994; Lien et al., 2007; Schwean-Lardner et al., 2012a, 2016; Yang et al., 2015a; Olanrewaju et al., 2015; Zhai et al., 2016). All birds were given 23L exposure during first week of their life span, as expected, there were no significant differences observed for any parameter at this age. As expected, after photoperiodic treatment implementation post 7 d of age, photoperiod ($\geq$ 18L) significantly increased body weight at 26 d and 36 d of broiler chicken age which agrees with the work of Yang et al. (2015a) and Schwean-Lardner et al. (2016). Yang et al. (2015a) experiment

investigating photoperiod (12, 14, 16, 18, 20, 22 and 24 h per day) exposure effect at 35, 49, 63, and 80 d in medium growing broilers, demonstrated increased body weight with increased photoperiod expsosure. Moreover, higher final body weights were recorded when broilers were exposed to ≤ 20L photoperiod instead of ≤ 14L (Yang et al., 2015a). In support, in a 32 and 39 d old broilers study, Schwean-Lardner et al. (2012a) observed 14L raised birds were the lightest and 20L achieved the heaviest body weight, while 17L and 23L birds were intermediary, with similar weights. However, several studies have reported compensatory growth in older birds (> 40 d) when raised under short or restricted photoperiod so that growth rate was identical or superior to 23L reared (Downs et al., 2006; Lewis et al., 2009; Schwean-Lardner et al., 2016).

A reduction in feed consumption by birds under shorter photoperiods was suggested as a reason for the observed decrease in their body weight, as broilers mostly eat during the photophase of a lighting program (Olanrewaju et al., 2006; Brickett et al., 2007; Lien et al., 2007; Lewis et al., 2009; Schwean-Lardner et al., 2012a, 2016). Comparable to our results at all ages, broilers exposed to longer photoperiod lengths consumed more feed than birds given a shorter daylength (Brickett et al., 2007; Lien et al., 2007; Lewis et al., 2009; Schwean-Lardner et al., 2012a), however, differences between treatments becomes smaller as birds grew older (Lien et al., 2007). In the current research, broilers raised under the short photoperiod (13L), despite attaining a significantly lower body weight at 36 d of age, revealed improved feed efficiency when compared to birds under 18L:6D and 23L:1D, which agree to reports by Classen et al. (2004). No effect of dietary amino acid on feed consumption was detected in the current study, which agrees with an experiment where the effects of two dietary amino acids (lysine and methionine) were examined over 21 to 42 d of age in Ross 708 male broilers (Zhai et al., 2016)**.** However, other research (Kidd et al. 2004, Corzo et al., 2005) have reported an increase in feed intake when birds were fed low amino acid dense diets. This could be due to difference in their amino acid levels, types of birds used and age when this parameter was examined.

Both photoperiod and dietary amino acid levels significantly affected feed efficiency (mortality uncorrected and corrected), with shorter photoperiod and incremental

levels of dietary amino acids increasing feed efficiency. Coherent to our findings, Kidd et al. (2004), Corzo et al. (2005) and Lilly et al. (2011) have indicated increasing levels of dietary amino acids improve growth and also improve feed efficiency. Additionally, agreeing to Schwean-Lardner et al. (2012a, 2016) work, the current study demonstrated that increasing photoperiod negatively affected both feed efficiencies (G:F and G:F^{m}), during 29-36 d period and overall between 7-36 d of age. The improved feed efficiency recorded here under shorter photoperiod (13L) could be due to lower energy expenditure with nightly metabolic rates (Hassanzadeh et al., 2016), lower physical activity (Schwean-Lardner et al., 2012b, 2016), reduced heat production, (Buyse et al., 1993), better nutrient digestion (Svihus, 2014), lower incidence of mortality (Hassanzadeh et al., 2012) and melatonin secretion reaching peak during night acting via endocrine system on metabolism (Osei et al., 1989; Clark and Classen, 1995; Apeldoorn et al., 1999; Schwean-Lardner et al., 2016). Moreover, it has been suggested that normal feeding behavior can be typically exhibited by birds maintaining a diurnal rhythm, only when exposed to sufficient (4-6 h) darkness exposure (Olanrewaju et al., 2006; Schwean-Lardner et al., 2013). In general, the effect of photoperiod on gender was recorded here, at all time points, females ate less, had lower body weight and lower feed efficiency, than their male counterparts, which is in agreement to Schwean-Lardner et al. (2012a). In agreement to the current study's findings, earlier studies have shown a gender specific effect of dietary amino acid levels, revealing that in contrast to their female counterparts, males were heavier, more feed efficient but had less proportional breast tissue (Corzo et al., 2005, Kidd et al., 2004; Brickett et al., 2007).

Both photoperiod and dietary amino content independently influenced breast yield which was in close agreement with Renden et al., (1994). Lower proportionate breast yield after exposure to 13L versus ≥18L, as observed in the current study, was in agreement to broiler studies conducted by Brickett et al., (2007), Lien et al., (2007) and Mlaba et al., (2015). Supplying higher levels of dietary amino acid levels to broiler chicken significantly increased breast yield here, agreeing to previous findings (Hickling et al., 1990; Kidd et al., 2004; Corzo et al., 2005; Zhai et al., 2016). Lysine (Kidd et al., 2004) and methionine

(Hickling et al., 1990), have been considered the most critical amino acid for breast meat yield and building blocks for protein accretion in body. A positive effect on carcass weight has also been identified recently, by increasing levels of these essential amino acids in commercial broilers diets (Zhai et al., 2016).

In agreement to previous experiments (Buyse et al., 1993; Cutler et al., 2005; Duve et al., 2011), our results revealed that a broiler's relative crop weight (percent g/g bwt) increased under the short photoperiod exposure (13L) compared to 18L and 23L. This was mainly due to these birds storing more feed prior to long dark period (Buyse et al., 1993; Duve et al., 2011). Here, starting from a young age (12 d), broilers housed under short photoperiod (13L) demonstrated an extensive use of their crop for feed storage which is in agreement with Lewis et al. (2009), where within 5 d post-installation of the photoperiod treatments, broilers genotypes exhibited altered feeding behavior, storing feed in crop, when exposed to ≤15L photoperiod. Birds housed under 18L and 23L, did not exhibit this extensive feed storage behavior, as evidenced by their smaller crop weight, which was in agreement to the suggestion that the crop is used as food storage organ only in birds exposed to shorter photoperiods, while birds exposed to extended photoperiods, usually exhibit less crop utilization (Svihus et al., 2010; Duve et al., 2011). A lack of an effect of amino acid level on crop weight and feed consumption, in conjunction with a strong positive effect of photoperiod on crop weight and feed consumption, in the present study, supports the speculation that photoperiod could act as a more effective signal in influencing crop size mainly due to a change in feeding behavior.

This altered, extensive and extended, feed storage pattern in crop, in turn may affect crop microenvironment, assessed here by recording crop pH. Crop pH results of the current study were in close agreement to previously published chicken (Durant et al., 1999; Hinton et al. 2000a,b; Hilmi et al., 2007; Nishii et al., 2016) and turkey (Cutler et al., 2005) values, and ranged between 4–6. In the present study, the reason for the documented lack of photoperiod effect on crop pH in the 12 d old broilers might be due to the time required for birds to adapt their feeding behavior to the new photoperiod schedule, implemented at 7 d of age. At all data collection time points (12, 19, 26 and 33 d) in this study, compared to

18L and 23L, the lowest crop pH (4.74-5.14) values were observed in broilers housed under the 13L, reaching significance at 19, 26 and 33 d of age. This could be speculated to be due to increased feed storage in crop and higher synthesis of fermentation products by resident crop lactobacilli, reducing crop pH (Cutler et al., 2005). Although, a number of factors contribute to crop pH (Ptak et al., 2015), however, the abundance of crop lactobacilli has been well recognized for lowering pH due to organic acid production (Fuller, 2001). The organic acids produced by crop resident bacteria have a positive effect on the gut and its mucosal health, and can prevent colonization by harmful bacteria, such as *E. coli* and *Salmonella* spp. (Fuller, 1973; Hinton et al., 2000a,b; Hilmi et al., 2007; Fonseca et al., 2010; Ptak et al., 2015; Witzig et al., 2015). This effect was not only seen in the crop, but also in the caeca, spleen and liver of molting hens (Ricke, 2003).

Poultry intestinal microbiota plays an important role in sustaining health and productivity (Janczyk et al., 2009; Teirlynck et al., 2009a,b; Choi et al., 2014; Scanes and Pierzchala-Koziec, 2014) and recent advances in 16S rRNA gene based molecular techniques have enabled researchers to identify and characterize gut microbiota, independent of culture based requirements (Witzig et al., 2015; Zduńczyk et al., 2015, Saxena et al., 2016). T-RFLP is one such molecular technique, well established and commonly used to characterize different poultry GIT ecosystems (Witzig et al., 2015). The recorded discrepancies between the predicted and observed TRF lengths (±4 bps) of this study, were in agreement to earlier experiments using the T-RFLP technique for assessment of the microbiota community in various environments (Sakamoto et al., 2003). Moreover, the 16S rRNA gene based T-RFLP analysis of crop contents in the current study revealed an increased mean relative abundance of TRFs (177, 181, and 572-573 bp by Msp1), representing mainly beneficial lactobacilli in birds exposed to 13L compared to 23L photoperiod. Simultaneously, these birds also demonstrated a decreased abundance of TRFs (488 and 566 by Msp1), representing uncultured rumen bacteria and *Enterococcus faecalis*. These differences can be due to changes in broiler birds feeding pattern; noticed here by extensive utilization of the crop for feed storage in these birds which could provide substrates for resident lactobacilli bacterial population and extended feed residency (Cutler

et al., 2005). Therefore, it can be speculated that short photoperiods result in a higher abundance of *Lactobacillus* members due to feed storage. Altered feeding behavior, higher feed intake, by Soay rams exposed to 16L:8D (long) photoperiod regimes compared to 8L:16D (short), was suggested as the primary factor for observed differences in DGGE banding pattern investigating rumen (ciliate and bacterial) microbial diversity (McEwan et al., 2005). Research in other species; rat and rams, has also photoperiod could exert effect on GIT microbiota differences due to its potential to influence host autonomic nervous system, endocrine system, digestive system and metabolism (McEwan et al., 2005; Bailey et al., 2010; Walton et al., 2011; Cerdá et al., 2016). In male Siberian hamsters, bacterial tag-encoded FLX amplicon pyrosequencing of caecal microbiota revealed in contrast to 8L:16D (short) photoperiod, animals housed under 16L:8D (long) photoperiod had a higher abundance of Proteobacteria phylum members which increased body and inguinal adiposity (Bailey et al., 2010).

The cluster analysis of crop content T-RFLP profiles, here, demonstrated grouping according to the photoperiod and amino acid level. To the author's knowledge, no broiler chickens T-RFLP GIT microbial community assay, exposed to different photoperiod treatments, is present for comparison. But, in line with our work, dietary alteration of minerals (phosphorus and calcium) have shown to cluster differently for the broiler chicken crop (Witzig et al., 2015).

The qPCR data results were in agreement to the T-RFLP analysis, which indicated a greater relative abundance of *Lactobacillus* member species in birds exposed to the short (13L) versus long (23L) photoperiod. Furthermore, the broiler chicken crop qPCR results, in this study, for total bacterial counts (10^9-10^{10} /g content) were in close agreement to previously published values for chicken (Guan et al., 2003; Hilmi et al., 2007; Hammons et al., 2010; Nishii et al., 2016). The current results also confirmed *Lactobacillus* as the predominant broiler chicken crop inhabitant, when reared under commercial conditions (Fuller, 2001; Hammons et al., 2010; Scanes and Pierzchala-Koziec, 2014; Witzig et al., 2015). It has been established that across different avian GIT segments, crop has the highest diversity of *Lactobacillus* member species and selected *Lactobacillus* species enumerated

here in current work, has been previously reported from broiler chicken crops (Guan et al., 2003, Gong et al., 2007; Hilmi et al. 2007; Witzig et al., 2015). Remarkably, short photoperiod (13L) resulted in a higher abundance of *Lactobacillus* species especially *L. gallinarum.* Earlier studies have indicated increase in *Lactobacillus* after short photoperiod exposure versus long (McEwan et al., 2005; Bailey et al., 2010; Walton et al., 2011), but what promoted *L. gallinarum* only in the current study, needs further investigations.

Crop pH data (33d) representative of fermentation activity by resident bacterial population (Cutler et al., 2005), correlates well with the qPCR data obtained, supporting higher abundance and lower crop pH under short photoperiod exposure. Dietary amino acid levels did not influence crop microbial community members in particular, in this study which agrees to Hammons et al. (2010) work where minor variations in broiler diets (corn-soy versus corn-soy with wheat middlings with lysine) did not affected crop inhabitant *Lactobacillus* species. Moreover, feeding graded levels (100, 110, 120 and 130 % above requirement) of threonine in 42 d old Ross 308 broiler chickens had no influence on ileum and ceacal microbial counts (*E. coli* and *Lactobacillus*) assessed by culturing (Eftekhari et al., 2015). The qPCR data analyzed for dietary amino acid effect, however, did not correlate with T-RFLP and low crop pH data noticed in this current experiment. The small sample sizes and small differences in amino acid levels in the current study might not have identified the statistical significance in crop microbiota investigating differences for photoperiod and amino acid level. However, future studies with larger numbers of birds, short chain fatty acid analysis in conjugation of advance molecular profiling techniques would help in revealing an effect of photoperiod or dietary manipulations on the crop's microbiota in better detail.

Typically, *Lactobacillus* have long been identified, as having a positive role on the gut immune system through the prevention of its colonization by pathogenic bacteria, which improves bird performance and health (Fuller, 2001; Scanes and Pierzchala-Koziec 2014; Zduńczyk et al., 2015). This might be the reason for recorded lower infectious mortalities with reduced photoperiod in current experiment (Chapter 2) and in agreement with Schwean-Lardner et al. (2013). This possibly can be attributed mainly due to increased

melatonin secretion, decreased physiological stress and improved immune function (Abbas et al., 2008; Schwean-Lardner et al., 2013, 2014). In support, Zamanzad-Ghavidel et al. (2011) found that broilers supplemented with Lactobacillus based probiotics (@1000 gram /1 ton diet) had a significantly reduced number of mortalities, in comparison to non-supplemented birds. Therefore, it can be suggested that darkness exposure might be acting via another mechanism, crop microenironment and microbial abundance manipulation, to affect intestinal health and disease prevention (Fuller, 2001; Hinton et al., 2000a,b; Cutler et al., 2005; Scanes and Pierzchala-Koziec 2014; Zduńczyk et al., 2015).

It has also been reported that broiler chickens with lower feed efficiency had reduced abundance of *L. acidophilus* and *L. salivarius* (de Lange and Wijtten, 2010). The qPCR enumeration of ileal *L. salivarius* in broilers revealed that a lower abundance of this bacterium was associated with better weight gain; postulated to a decrease in deconjugation of bile salts in the small intestine mediated by this organisms (Harrow et al., 2007). This is in close agreement to the current findings: a greater abundance of 177 bp TRF representing mainly *Lactobacillus* group members, could be potentially associated with a negative effect on 33 d old broiler chicken body weight. Recently, (Stanley et al., 2016) reported higher abundance of caecal *Lactobacillus* was associated with increased feed intake and poor feed efficiency in 25 d old broiler chickens. However, which *Lactobacillus* species are associated with poor feed efficiency, this need further studies.

Both diet and management stressors for broilers, investigated earlier, have shown potential to alter the gastrointestinal microbiological composition (Zduńczyk et al., 2015), thereby affecting growth, feed conversion, and health. However, in the current study, feed consumption and crop parameters were not influenced by amino acid levels, while photoperiod has shown to significantly affect feed consumption, crop size, pH and microbial ecology. Therefore, it can be speculated that photoperiod acts as a strong stimulus for feed storage in poultry, which in turn can affect native microbiota (Lewis et al., 2009; Cutler et al., 2005), differing in potential to harvest energy from the consumed diet (Bailey et al., 2010). Moreover, as dietary amino acids levels were not markedly different from industry (Aviagen) recommendations and we have a limited number of

replicates (total n=20) to investigate dietary amino acid effect in the current study, this could have led to lack of any noticeable differences. The data from this study indicate elevated numbers of predominantly *Lactobacillus* species in the crop, as analyzed by T-RFLP and qPCR, of broilers reared under a short (13L) versus long (23L) photoperiod exposed birds. However, photoperiod induced crop resident bacterial activity, here did not reveal any influence on the broiler's dietary amino acid requirements, but this area may benefit from additional attention.

3.6 Conclusions

The findings of this study suggest supplementing medium to high rather than low dietary levels of amino acid levels could improve broiler chicken growth parameters, feed efficiency and breast yield. Longer photoperiod exposure increased broiler chickens breast yield, however, 18L and 23L photoperiod exposure resulted in identical final weight, average weight gain and decreased feed efficiency in comparison to the 13L treatment. Compared to 18L or 23L, exposure of broiler chickens to 13L increased crop size, reduced crop pH and increased abundance of *Lactobacillus* species in the crop. Polymorphism demonstrated by T-RFLP profiles of the crop, indicated a difference in the resident bacterial communities between birds raised under the different photoperiod schedules (13L versus 23L), although it was difficult to identify which bacterial groups lead to the differences in the crop microbiota profiles by T-RFLP profiles analysis only. Additionally, a potential negative correlation was demonstrated between greater crop abundance of TRF representing Lactobacillus and the body weight of broilers. Detailed 16S rRNA gene based qPCR investigated of predominant broiler chicken crop inhabitant bacterial targets revealed high abundance of total, Lactobacillus and *L. gallinarum* in 13L exposed compared to 23L. This study further strengthens the premise that dietary amino acids mainly affected performance and breast yield, however, exposure to darkness has beneficial effects on bird health, especially proximal intestinal health. Therefore, more research is warranted to find out how to use photoperiod to maintain a balance between broiler growth and a microbial healthy GIT.

4.0 EFFECT OF DIETARY INGREDIENT TYPE, FEEDING FREQUENCY AND SCREEN SIZE ON MICROBIAL ECOLOGY OF THE CROP AND ILEUM IN BROILER CHICKENS

4.1 Abstract

A broiler chicken experiment was conducted to investigate the effect of dietary ingredient (D) type, feeding frequency (F) and hammer mill screen size (S) on microbial ecology of the crop and ileum. For this, 96 day-old male broiler birds (Ross 308) were placed in 12 floor pens and fed mash diets formulated with one of three primary dietary ingredients (corn vs. wheat vs. pea), ground using one of two hammer mill screens (350 mm vs. 1000 mm), and offered at one of two feeding frequencies (*ad-lib* vs. three meals per day). A common starter diet was fed from 0-14 d after which (15-36 d) experimental diets were randomly allocated to pens. At day 36 of the experiment, body weight of all birds was recorded before birds were euthanized to permit collection of crop contents, crop mucosa and ileal contents. Genomic DNA was extracted from contents and mucosa for analysis of microbial communities using 16S rRNA gene-based terminal-restriction fragment length polymorphism (T-RFLP) and quantitative polymerase chain reaction (qPCR) techniques. Final body weight of broiler chickens was not affected by dietary ingredients and screen size, however meal-fed broilers had the lower body weight in contrast to their *ad-lib* fed counterparts. Meal feeding significantly increased relative size of the crop and amount of recovered contents compared to *ad-lib* fed broiler birds. Moreover, the amount of crop contents also increased with coarsely ground diets. Analysis of T-RFLP profiles revealed discrete segregation of broiler crop contents, crop mucosa and ileal contents suggesting distinct location-specific microbial populations. Further, T-RFLP microbial profile analysis for crop contents and mucosa revealed that broilers fed pea diets segregate from broilers fed corn or wheat diets. Moreover, feeding of corn or wheat based

diets in broiler chickens increased the diversity indices of the crop mucosa-associated bacterial community, compared to pea-based diets. Quantitative PCR analysis of crop content revealed a higher abundance of total bacteria, *Lactobacillus* spp., *L. johnsonii* and *L. salivarius* in broilers fed corn and pea-based diets while in crop mucosa pea diets promoted the increased abundance of *L. gallinarium* versus corn and wheat-fed birds. No clustering of T-RFLP profiles generated from ileal contents was observed, however, in contrast to crop, total *Lactobacillus* spp. genome count decreased with inclusion of pea. Three times per day meal feeding increased ileal counts of *L. johnsonii*, *L. gallinarium* and *L. reutri* compared to *ad-lib* fed broiler birds. In conclusion, we demonstrate effects of feed ingredients, processing and management on microbial composition of the crop which could have implications for broiler gut health.

4.2 Introduction

Use of antibiotic growth promoters (AGPs) in the poultry industry has been linked to better gut health acting primarily through alteration of intestinal microbial community (Yegani and Korver, 2008). As a result, numerous studies have investigated the effect of dietary and management strategies on microbial community composition with the intention of promoting beneficial microbial communities and improved intestinal health (Yegani and Korver, 2008; Roberts et al., 2015). While much of the focus has been on the small intestine and cecum, few investigators have considered the importance of crop microbial communities (Classen et al., 2016). In poultry, the largest and most dense bacterial populations are found in two GIT locations, the crop, an out pouching of the oesophagus in the upper tract, and the ceca, paired blind sacs near the end of the tract (Rehman et al., 2007; Scanes and Pierzchala-Koziec, 2014). Moreover, it has been suggested that compared to highly the diverse and differentiated microbial community of the caeca, the poultry crop has a distinct predominance of *Lactobacillaceae* members (Scanes and Pierzchala-Koziec, 2014; Witzig et al., 2015; Saxena et al., 2016). As the first organ to be encountered by enteric pathogens, the crop microbiota has been considered to play a significant role in prevention of colonization of bacteria such as *Salmonella* (Hinton et al., 2000a; Scanes and Pierzchala-Koziec, 2014). Furthermore, crop microbiota may also serve

as an inoculum for the distal intestine affecting distal tract microbial composition (Hilmi et al., 2007).

Under common management practices used in commercial broiler production the crop is often not actively utilized, particularly when raised under continuous (24L:0D) or near continuous (23L:1D) photoperiod (Buyse and Decuypere, 2003) or when allowed *ad-lib* feed access (Svihus, 2014). Moreover, it has been suggested that feed withdrawal in molting hens, could lead to change in their crop microenvironment; decreased *Lactobacillus* population and increased pH (Durant et al., 1999) contributing to higher *Salmonella* prevalence in digesta and translocation to tissues.

Several studies have suggested a strong effect of dietary ingredients and screen size (Engberg et al., 2002; Rehman et al., 2007; Yegani and Korver, 2008) on distal gut microbial communities. For instance, *Salmonella typhimurium* and *Clostridium perfringens* counts decreased in distal intestinal tract (ileum and caeca) of broiler chickens fed whole wheat (Yegani and Korver, 2008). Similarly, feeding maize-soybean diets significantly decreased caecal *Salmonella* counts in broiler chickens versus wheat/rye-soybean fed birds (Teirlynck et al., 2009b) possibly due to the wheat/rye-based diet inducing an inflammatory bowel type of condition, mucosal damage and an alteratered caecal microbiota. In contrast to the ileum and caeca, very few studies have extensively investigated broiler chicken crop microbiota under different dietary regimes using molecular based techniques (Guan et al., 2003; Hilmi et al., 2007). In addition, information is still lacking for variety of common dietary ingredients such as pea.

Several *in-vitro* studies have indicated that bacteria at intestinal sites fulfill their growth requirement mainly from simple sugars and peptides, and have been shown to compete with the host for ingested dietary substrates (Langlands et al., 2004; Shakouri et al., 2009; Pan and Yu, 2014). Therefore, in comparison to dietary fibre and other dietary constituents primarily linked with distal gut microbial community composition, we hypothesize that readily available dietary nutrients (e.g. starch) and feeding frequency are major factors affecting crop bacterial community composition. Moreover, screen size has the potential to markedly affect the surface area for enzymatic action and microbial access.

Therefore, a 16S rRNA gene-based analysis of crop and ileum microbial communities in broiler chickens was conducted to determine the effect of major feed ingredients, screen size and feeding frequency.

4.3 Materials and Methods

4.3.1 Experimental Design and Bird Management

At total of 96 day-old Ross 308 (Lilydale Hatchery, Wynyard, Saskatchewan) male broiler birds were placed in 12 floor pens at Animal Care Unit at University of Saskatchewan and assigned to dietary treatments in a 3X2X2 factorial arrangement. Treatments included feeding of mash diets formulated on one of three primary dietary ingredients (corn, wheat or pea), and ground using a hammer mill using a using a 350 mm (fine) or 1000 mm (coarse) screen-hole size. Diets were offered in one of two feeding frequencies (*ad-lib* vs. three meals per day at 08:00, 13:00 and 17:00pm). The photoperiod during the experimentation was 23L:1D with lights on at 08:00 h. Birds were fed with a common non-medicated commercial corn and soy based starter diet for the first 14 d followed by experimental grower diets (Table 4.1) offered from 14 to 36 d of age. All diets had similar energy and crude protein levels, however, energy and amino acids (threonine, methionine + cysteine) values were approximately 5 % below Aviagen Broiler Nutrition Specification (2007) for the Ross 308 broiler grower diet, in order to adjust to high amounts of pea. Wheat and pea diets were exclusively supplemented with a commercial enzyme cocktail (Table 4.1) for plant cell wall degradation.

At 36 d of age and beginning at 09:00 h; all birds were killed in random order with respect to treatment. After euthanasia, birds were weighed and the digestive tract was removed to permit isolation of the crop and ileum (Meckels to 2 cm proximal to the ileo-caecal junction). For the isolated crop, all content was removed using gentle pressure followed by blotting and weighing. A subsample of contents was removed for microbial analysis and the pH (Beckmann Φ50 pH meter, CA, USA) of the remaining crop content was determined after mixing with 9 volumes of ddH_2O. After emptying of content, the crop was longitudinally dissected and washed three times with distilled water and two times

with sterile 0.1 % PBS-T (0.1 % Tween-20 in 1X PBS). Mucosal samples were subsequently collected by gentle scrapping of the mucosal surface using sterile glass slide (modification of Ruiz et al., 2015). Ileum content was expelled from the distal 2/3 of the tissue using gentle pressure. All intestinal contents and mucosa were stored at -80 °C until analysis.

Table 4.1. Ingredient composition (%) and nutrient content of experimental broiler chicken diets including calculated and analyzed nutrient concentration.

Ingredients (%)	Corn	Wheat	Pea
Corn	58.39	------	------
Wheat	------	70.48	10.00
Pea	------	------	68.00
Soybean Meal	32.91	21.00	7.40
Canola Oil	1.00	1.91	6.87
Fish Meal (Herring)	2.37	1.00	2.56
Dicalcium Phosphate	1.92	1.62	1.58
Limestone	1.04	1.17	1.09
DL-Methionine	0.30	0.27	0.41
L-Lysine HCl	------	0.40	------
L-Threonine	------	0.06	------
Indigestible Marker (Celite®[1])	1.00	1.00	1.00
Vitamin/Mineral Premix[2]	0.50	0.50	0.50
Salt (NaCl)	0.47	0.44	0.43
Choline Chloride	0.10	0.10	0.10
Enzyme[3]	------	0.05	0.05
Calculated Nutrient Concentration			
Crude Protein (CP)	22.0	22.0	22.0
Calcium	0.95	0.90	0.90
Avail. Phosphorus	0.50	0.45	0.45
M.E. Poultry (kcal/kg)	2950	2950	2950
Methionine + Cysteine	0.93	0.94	0.90
Lysine	1.24	1.29	1.44
Tryptophan	0.27	0.28	0.22
Threonine	0.85	0.80	0.80
Isoleucine	0.93	0.85	0.89
Valine	1.08	0.99	1.06
Arginine	1.50	1.35	1.74
Sodium (Na)	0.22	0.22	0.22
Analyzed Nutrient Content (% DM basis) of main dietary ingredient			
Fat	3.75	1.37	0.99
Total Starch	68.19	62.30	46.06
Resistant Starch	1.71	0.97	3.33
Acid detergent fiber	3.87	3.37	8.69
Neutral detergent fiber	10.09	13.87	10.33

[1]Celite Corporation, Quincy, USA.

[2]Amount supplied per kilogram diet: vitamin A 9600 IU, vitamin D (D3) 3000 IU; vitamin E 25 IU; menadione 1.5 mg; thiamine 1.5 mg; riboflavin 5 mg; pyridoxine 1.5 mg; vitamin B12 0.01 mg; niacin 30 mg; pantothenic 8 mg; folic acid 0.5 mg; biotin 0.06 mg; copper 10 mg; iron 80 mg; manganese 80 mg; iodine 160 mg; zinc 80 mg; selenium 0.3 mg; calcium carbonate 500 mg; antioxidant 0.62 mg.

[3]Avizyme ® 1302 (Danisco Animal Nutrition, United Kingdom) contain 5000 U/g endo-1,4-beta-xylanase and 1600 U/g subtililsin (protease).

4.3.2 Genomic DNA Extraction

Genomic DNA was extracted from digesta contents after some modification of method described by Dumonceaux et al. (2006). Briefly, digesta contents (350 mg) or crop mucosa (500 mg) were transferred into Bead-beater tubes (Mo-Bio Laboratories, Solano Beach, CA), containing 365 µL of buffer B1 (containing 0.5 % of Triton X-100 and Tween 80) with RNase A (10 mg/mL), 7.5 µL of lysozyme (100 mg/mL concentration) and 20 µL of proteinase K (20 mg/mL) and incubated for 1 h at 37 ºC. Samples were again incubated for 30 min at 50 ºC after mixing with 135 µL of buffer B2 (containing 675 mM of Guanidine-HCl and 4 % of Tween 20). Samples were then frozen at -70 ºC for 30 min and, after thawing, 700 µL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) was added. Samples were shaken three times for 20 s at speed setting of 5 in a bead beater instrument (Bio 101 ThermoSavant FP120). The top phase was separated after centrifugation at 14,600 X g for 15 min and mixed with equal volumes of chloroform. Top phase was again removed and mixed with 70 µL volume of 3M sodium acetate and 700 µL of isopropanol. After centrifugation (14,600 X g, 15 min) the resulting pellet was washed with 70 % ethanol, air dried, dissolved in 100 µL of UV-sterile water and stored at -20 °C. Before, proceeding for DNA extraction mucosal samples were diluted in 0.1 % PBS-T and centrifuged at 27000 X g for 15 min at 4 °C to pellet bacterial cells as adopted by Gong et al. (2007) for crop mucosa. Finally, the crop mucosa pellet was resuspended in 500 µL distilled water and processed as described for digesta.

4.3.3 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism (T-RFLP) analysis was performed on crop contents, crop mucosa and ileal digesta samples as described by Fernando et al. (2010). Briefly, PCR amplification of extracted DNA was carried out using 16S rRNA gene specific universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 926R (5'-CCGTCAATTCATTTGAGTTT 3') (Fernando et al., 2010). For ileum contents reverse primer R3 (TCTACGCATTTCAC; Dorsch and Stackebrandt, 1992) was used to amplify the 16S rRNA gene. The forward primer (8F) was labeled with 6'-

(carboxylfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide (6-FAM) at the 5' end. The reaction mixture (50 µL) contained 1 µL of template DNA, 5 µL of 10 X incubation buffer, 1.5 µL of 50 mM MgCl2, 1.3 µL of each primer (10 µM), 1.5 µL of each deoxynucleoside triphosphate (10mM), 0.2 µL of Taq polymerase (Invitrogen, USA) and 38.5 µL of UV-sterilized Millipore water (Pieper et al., 2009). Amplification conditions were 94 ºC for 5 min followed by 34 cycles of 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 1 min and final extension at 72 ºC for 7 min using an Ependorf Mastercyler EP (Hamburg, Germany). Confirmation of PCR amplification and product size was conducted by agarose gel electrophoresis (1X TAE buffer containing 0.5 µg ethidium bromide/mL of agarose) using 5 µL of the completed PCR reaction. QIAquick column purification kit (Qiagen, CA, USA) was used to purify PCR products of confirmed size, following the manufacturers' instructions. The DNA concentration of purified PCR products was measured by absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologie, Inc., Wilmington, DE). Purified PCR products (200 ng) were digested for 18 h at 37 ºC, using 20 U of Msp1 restriction enzyme (Fermentas, EU) in a 20 µL reaction volume. Duplicate aliquots of digested restriction product (2 µL) were mixed with 9 µL of Hi-Di^{TM} formamide (Genetic Analysis Grade, Applied Biosystems) and 0.5 µL of internal LIZ size standard (Applied Biosystems, CA, USA) before denaturing at 95 ºC for 2 min and immediate cooling on ice. Denature restriction product was them separated using an ABI PRISM 310 genetic analyzer (ABI Applied Biosystems, USA). Resulting T-RFLP profiles were imaged by GeneMapper software version 3.7 (Applied Biosystems, CA, USA). TRF size was estimated in reference to the internal standard using Local Southern method (± 3 bp), giving size in base pairs and peak area for each TRF (Fernando et al., 2010). TRFs below 50 bp and above 500 bp were eliminated from the analysis. Relative peak area for each TRF belonging to each sample was calculated by dividing its peak area by total peak area of that sample. Data was normalized by manually removing the TRFs below 1 % of relative peak area to avoid primer dimmer contamination or background noise (Pieper et al., 2009; Fernando et al., 2010).

T-RFLP profiles were analyzed using Bionumerics software version. 5.1 (Applied Maths, Austin, TX, USA). T-RFLP patterns were manually normalized and imported into the program (Fernando et al., 2010) after relative peak area for each TRF was transformed by arc(sin)√x. Dice similarity coefficients were calculated using area sensitivity and a dendrogram was constructed by the unweighted pair group method (Fernando et al., 2010). Evenness (Margalef, 1957), Shannon-wiener (Shannon and Weaver, 1949), and Simpson (Simpson, 1949) diversity indices were also determined. Moreover, to further examine any prospective grouping of microbial communities according to treatment (ingredients, feeding frequency, feed size) and/or location used in this study, principal component analysis (PCA) of crop content, crop mucosa and ileum T-RFLP profiles was also conducted in Bionumerics 5.1 software (Wang et al., 2004).

4.3.4 Quantification of Selected Predominant Bacterial Groups and Species

The qPCR assays for quantification of selected predominant bacterial target groups and species were conducted by using iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories Inc., CA, USA) in CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, Canada). The standards for qPCR enumeration assay for each bacterial species were made using PCR amplification of genomic DNA extracted from type strains (ATCC, Table 4.2) (Fernando et al., 2010). Purified PCR product (QIAEX II, Qiagen) was than quantified using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologie, Inc., Wilmington, DE) and a 10-fold serial dilution was used to construct standard curves based on copy number as calculated using the formula:

Number of copies = [DNA (pg) * 6.022 x 1023 (copies per mole)] / [fragment length (bp) * 1x1012 (pg / g) * 650 (g / mole of bp)]

A 25 µL reaction mix contained 12.5 µL Platinum SYBRGreen qPCR Supermix UDG (Invitrogen, Carlsbad, CA, USA), 0.8 µM of each primer, calibration standards or 2 µL of template DNA mixed in 6.5 µL of double distilled sterilized water. The amplification program for any bacterial target consists of following steps: 95 ºC for 3 min followed by 40 cycles of 95 ºC for 40 s, and the bacterial species specific annealing temperature (Table 4.2) for 40 s. The *L. gallinarum* (ATCC 33199) PCR primers were designed during this

study, using cpn60 UT database (http://www.cpndb.ca/) sequence and Beacon Designer software (Premier BioSoft International, Palo Alto, CA).

Table 4.2. Primers used for detection and enumeration of selected predominant bacterial groups and species in upper broiler gastro-intestinal by PCR and qPCR in this study.

Bacterial Target	Amplicon size (bp)	Annealing temp. (°C)	Sequence (5'-3')	Reference
Total bacteria[1]	200	60	F-CGGYCCAGACTCCTACGGG R-TTACCGCGGCTGCTGGCAC	Lee et al., 1996
Streptococcus spp[1].	485	57	F-GTTAGCCGTCCCTTTCTGG R-GAGTTTGATCCTGGCTCAG	Franks et al., 1998
Bifidobacterium spp.[1]	550-563	56	F-CTCCTGGAAACGGGTGG R-GGTGTTCTTCCCGATATCTACA	Matsuki et al., 2002
Total *Lactobacillus*[1]	346	55	F-GCAGCAGTAGGGAATCTTCCA R-GCATTYCACCGCTACACATG	Walter et al., 2001
L.crispatus[2]	125	59	F-TCCTTACATTTTGATCACTGA R-GAGCTTCACCAGTAACGTC	Dumonceaux et al., 2006
L. johnsonii[2]	146	55	F-TACTATTGAAGAATCAAAGGG R-TCAGTAATCAAAATGTAAGGG	Dumonceaux et al., 2006
L. salivarius[1]	332	60	F- CGAAACTTTCTTACACCGAATGC R- GTCCATTGTGGAAGATTCCC	Feng et al., 2010
L. reutri[1]	305	58	F-CAGACAATCTTTGATTGTTTAG R-GCTTGTTGGTTTGGGCTCTTC	Feng et al., 2010
L. gallinarum[1]	167	58	F- GCTAACCCAGTTGGCATTCG R-TCCATAGCGTCAGCAATCAAAG	Designed in this study

[1]16S rRNA gene based primers; [2]cpn-60 gene based primers.

4.3.5 Statistical Analyses

Statistical analyses was conducted using a 3X2X2 factorial arrangement of treatments (main effects and interactions) for all collected data using the Proc Mixed procedure of SAS (SAS 9.1) where means were separated using Tukey's multiple range test. For feed intake, pen was the experimental unit and no statistical comparisons were possible. For all other measurements individual birds were the experiment unit. The relative empty crop weight data was log transformed using Log+1, prior to statistical analysis. Differences were considered significant when $P \leq 0.05$.

4.4 Results

4.4.1 Body Weight and Crop Parameters

Body weight of male broiler birds at 36 d of age was not influenced by the dietary ingredient type (Table 4.3). Birds fed *ad-lib* were heavier as compared to meal fed counterparts ($P<0.02$) while screen size did not affect final body weight. Feed intake recorded from 29-36 d of broilers age, ranged from 185 g/bird to 199 g/bird and was not significantly affected by any treatment used in this study. However, birds fed pea diets consumed the greatest (199 g/d) followed by corn (190 g/d) and wheat (185 g/d). Meal-fed birds consumed more feed (196 g/d) compared to birds fed *ad-lib* (187 g/d) while fine ground diet-fed birds consumed 190 g/d compared to 193 g/d consumed for birds fed coarse ground diets. As expected, relative weight of the crop and the wet weight of crop contents were significantly increased by meal feeding as compared to *ad-lib* (Table 4.3). Wet weight of crop contents was also increased when a coarsely ground diet was fed. No effect of treatment on pH of crop content was observed in present study.

Table 4.3. Effect of different dietary ingredient type, feeding frequency and screen size on body weight and crop parameters of 36 d old broiler chicken (n=48).

Treatment	Body weight	Crop		
		Relative weight (% of bwt)	Total wet digesta weight (g)	pH
Dietary ingredient (D)				
Corn	2019	0.36	21.21	5.62
Wheat	2052	0.34	20.53	5.64
Pea	1867	0.38	22.81	5.45
P-value	0.12	0.27	0.61	0.25
Feeding Frequency (F)				
Ad-lib	2071[a]	0.31[b]	17.02[b]	5.59
Meal Fed	1888[b]	0.41[a]	26.17[a]	5.54
P-value	0.02	<0.001	0.009	0.59
Screen Size (S)				
Fine	1940	0.35	15.52[b]	5.59
Coarse	2019	0.36	27.26[a]	5.56
P-value	0.31	0.46	0.0023	0.68
Interactions[1]				
D X F	NS	NS	NS	NS
SXF	NS	NS	NS	NS
DXS	NS	NS	NS	NS
DXFXS	NS	NS	NS	NS
Pooled SEM	41.06	0.01	2.48	0.07

SEM-Standard error of the mean.
[1]D= dietary ingredient, F=feeding frequency, S=screen size.
NS = Non-significant.
[a,b]Means with the same letter are not significantly different in the same column. P-values were considered significant at P<0.05.

4.4.2 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism (T-RFLP) profile analysis revealed clustering mainly based on dietary ingredient type (Figure 4.1, 4.2, 4.3). For both crop contents and mucosa, T-RFLP analysis revealed that crop microbiota from pea-fed birds segregate from corn and wheat -fed broilers (Figure 4.1 and 4.2). In contents, the pea cluster was observed only when fed as meals, whereas no obvious clustering of pea profiles was observed for *ad-lib* feeding. For crop mucosa, the pea cluster did not contain all birds and was not associated with feeding frequency. In the case of broiler chicken ileum, in contrast to proximal locations, no prominent clustering was observed associated with the primary dietary ingredient (Figure 4.3). In addition, among all three broiler chicken locations examined (crop content, crop mucosa and ileum content), T-RFLP cluster analysis revealed no influence of feeding frequency or grinding.

The three dominant components identified by PCA for each location explained over 80 % of sample variation (Figure 4.4) and confirmed clustering of pea-based diets in crop contents (Figure 4.4a) and to a lesser extent in crop mucosa (Figure 4.4b). No cluster was observed by PCA analysis of ileal contents (Figure 4.4c). Not surprisingly, the PCA analysis of T-RFLP banding patterns together revealed clustering on basis of GIT location (Figure 4.4 d).

The diversity indices calculated from T-RFLP profiles for crop content, crop mucosa and ileum contents are shown in Table 4.4. Diets based on pea reduced ($P< 0.05$) evenness as well as Shannon and Simpson indices values for crop mucosa microbial communities compared to communities when corn and wheat-based diets were fed. However, no other treatments affected evenness or diversity values for the communities studied.

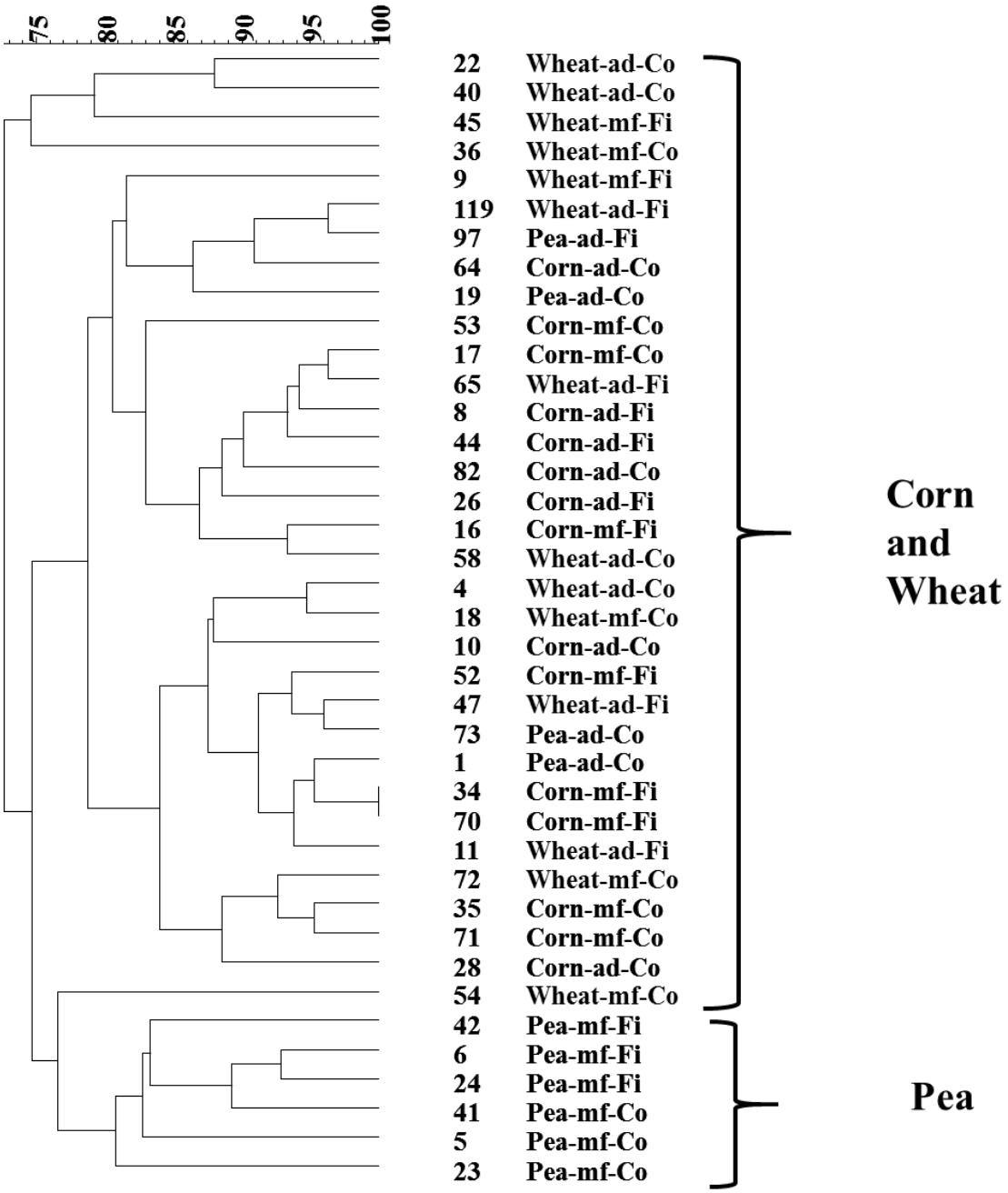


Figure 4.1. Cluster analysis of T-RFLP banding pattern of 36 d old broiler chicken crop (n=39) content fed three different dietary ingredient type (corn, pea and wheat), at *ad-libitum* (ad) or meal fed (mf) frequency grounded to fine (fi) or coarser (co) sizes. Clustering was based on the Dice similarity coefficient values and the unweighted pair group method using arithmetic averages (UPGMA), only samples values greater than 50 % similarity were shown here. The coefficient scale bar shown above in the diagram designates relative similarity between samples.

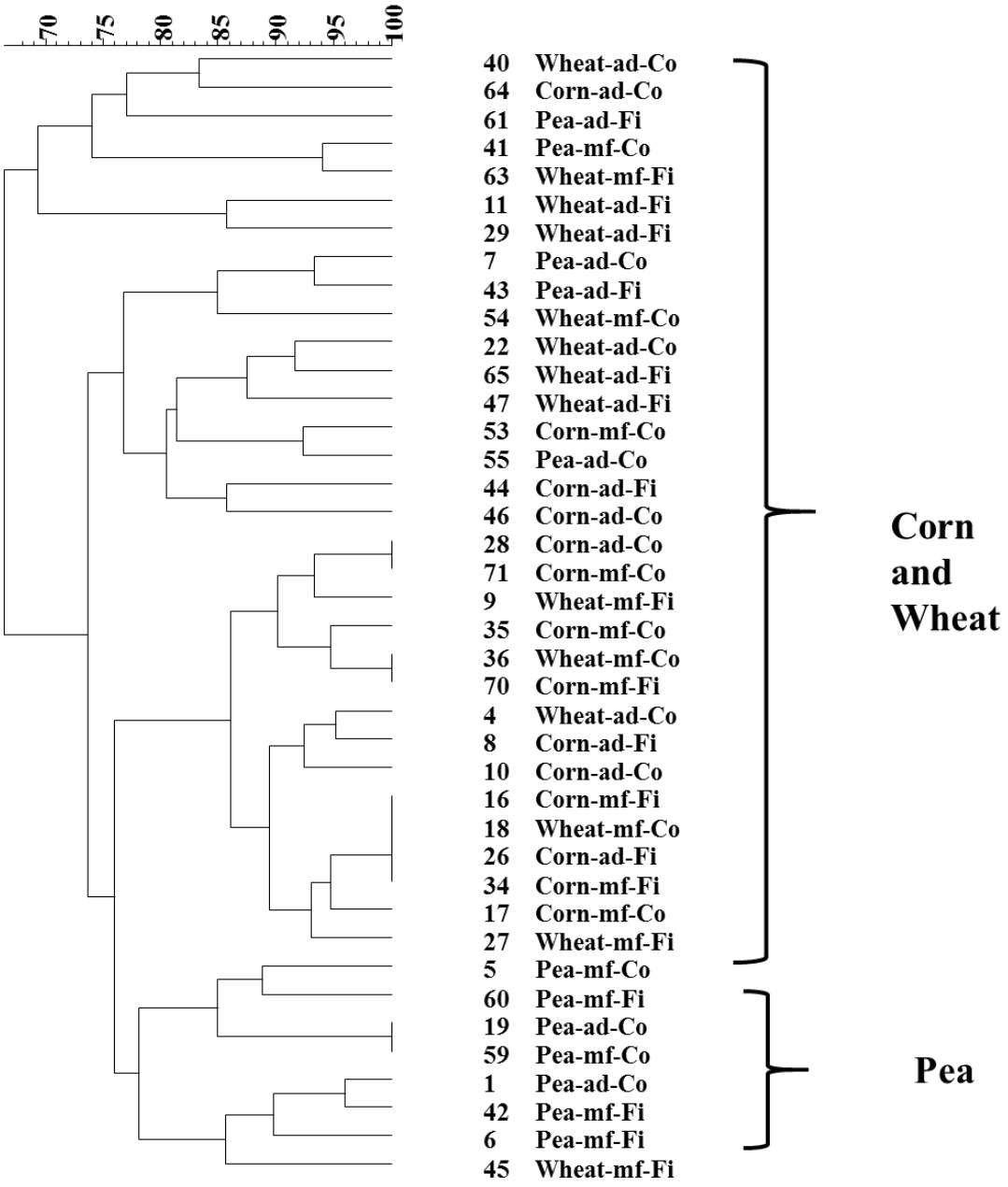


Figure 4.2. Cluster analysis of T-RFLP banding pattern of 36 d old broiler chicken crop mucosa (n=44) fed three different dietary ingredient type (corn, pea and wheat) at *ad-libitum* (ad) or meal fed (mf) frequency grounded to fine (fi) or coarser (co) sizes. Clustering was based on the Dice similarity coefficient values and the unweighted pair group method using arithmetic averages (UPGMA), only samples values greater than 50 % similarity were shown here. The coefficient scale bar shown above in the diagram designates relative similarity between samples.

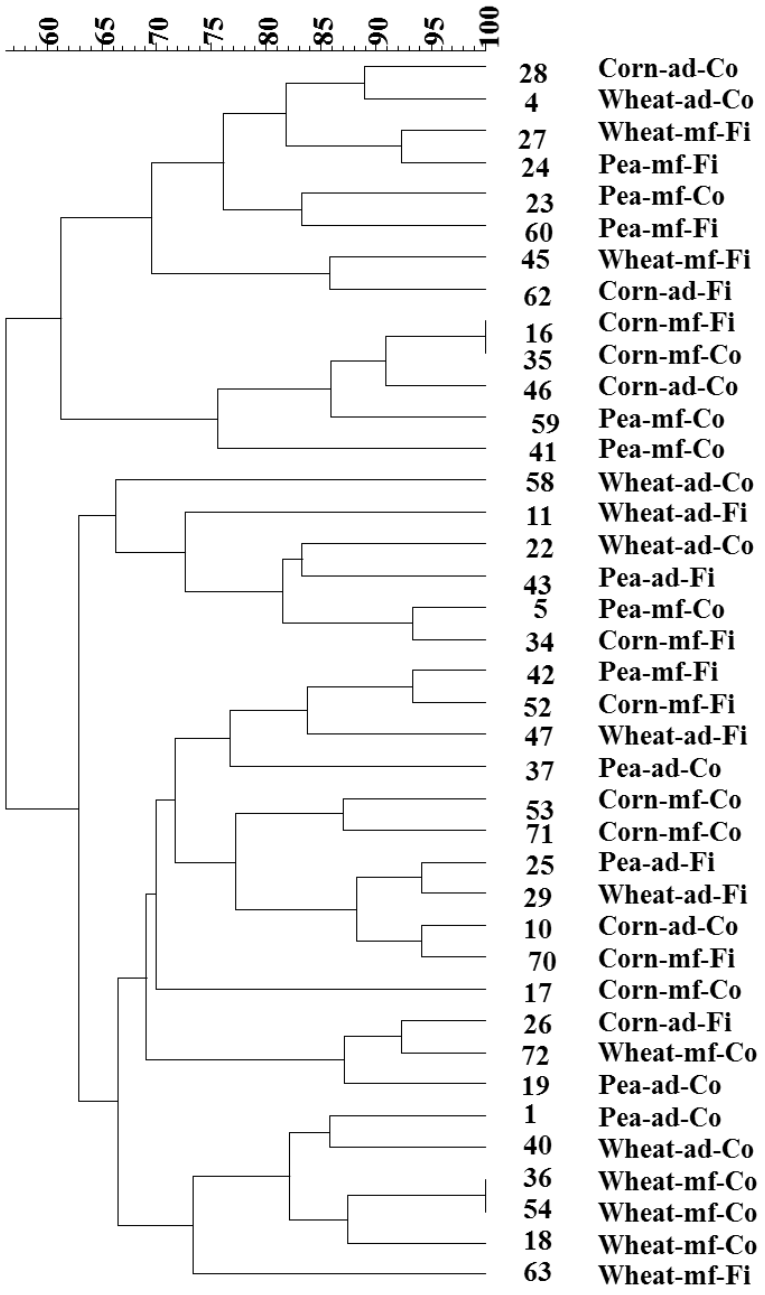


Figure 4.3. Cluster analysis of T-RFLP banding pattern of 36 d old broiler chicken (n=38) ileum content fed three different dietary ingredient type (corn, pea and wheat) at *ad-libitum* (ad) or meal fed (mf) frequency grounded to fine (fi) or coarser (co) sizes. Clustering was based on the Dice similarity coefficient values and the unweighted pair group method using arithmetic averages (UPGMA), only samples values greater than 50 % similarity were shown here. The coefficient scale bar shown above in the diagram designates relative similarity between samples

Table 4.4. Diversity indices calculated from T-RFLP profiles generated from crop content, crop mucosa and ileum contents of 36 d old broiler chickens fed different dietary ingredient type at *ad-lib* or meal-fed frequency grounded to fine or coarser sizes (n=48).

Location	Index	Dietary Ingredients (D)			Feeding Frequency (F)		Screen Size (S)		P-value[1]			Pooled SEM
		Corn	Wheat	Pea	*Ad-lib*	Meal- fed	Fine	Coarse	D	F	S	
Crop	Evenness	0.80	0.84	0.83	0.81	0.83	0.84	0.80	0.25	0.82	0.06	0.011
	Shannon	0.88	0.93	0.93	0.90	0.93	0.95	0.88	0.47	0.91	0.09	0.022
	Simpson	0.76	0.80	0.78	0.77	0.79	0.81	0.76	0.57	0.82	0.051	0.013
Crop Mucosa												
	Evenness	0.83[a]	0.77[a]	0.61[b]	0.75	0.73	0.72	0.76	0.001	0.77	0.42	0.027
	Shannon	0.84[a]	0.80[a]	0.61[b]	0.77	0.73	0.75	0.76	0.01	0.46	0.83	0.033
	Simpson	0.77[a]	0.73[a]	0.55[b]	0.69	0.68	0.67	0.70	0.003	0.74	0.57	0.029
Ileum												
	Evenness	0.65	0.68	0.73	0.64	0.72	0.67	0.69	0.51	0.19	0.79	0.029
	Shannon	0.50	0.49	0.51	0.46	0.54	0.47	0.53	0.93	0.23	0.29	0.029
	Simpson	0.55	0.56	0.59	0.52	0.61	0.54	0.60	0.87	0.16	0.37	0.030

SEM-Standard error of the mean.

[1]D=dietary ingredient, F=feeding frequency, S=screen size.

[a,b]Means in same column with different letters are different at $P < 0.05$.

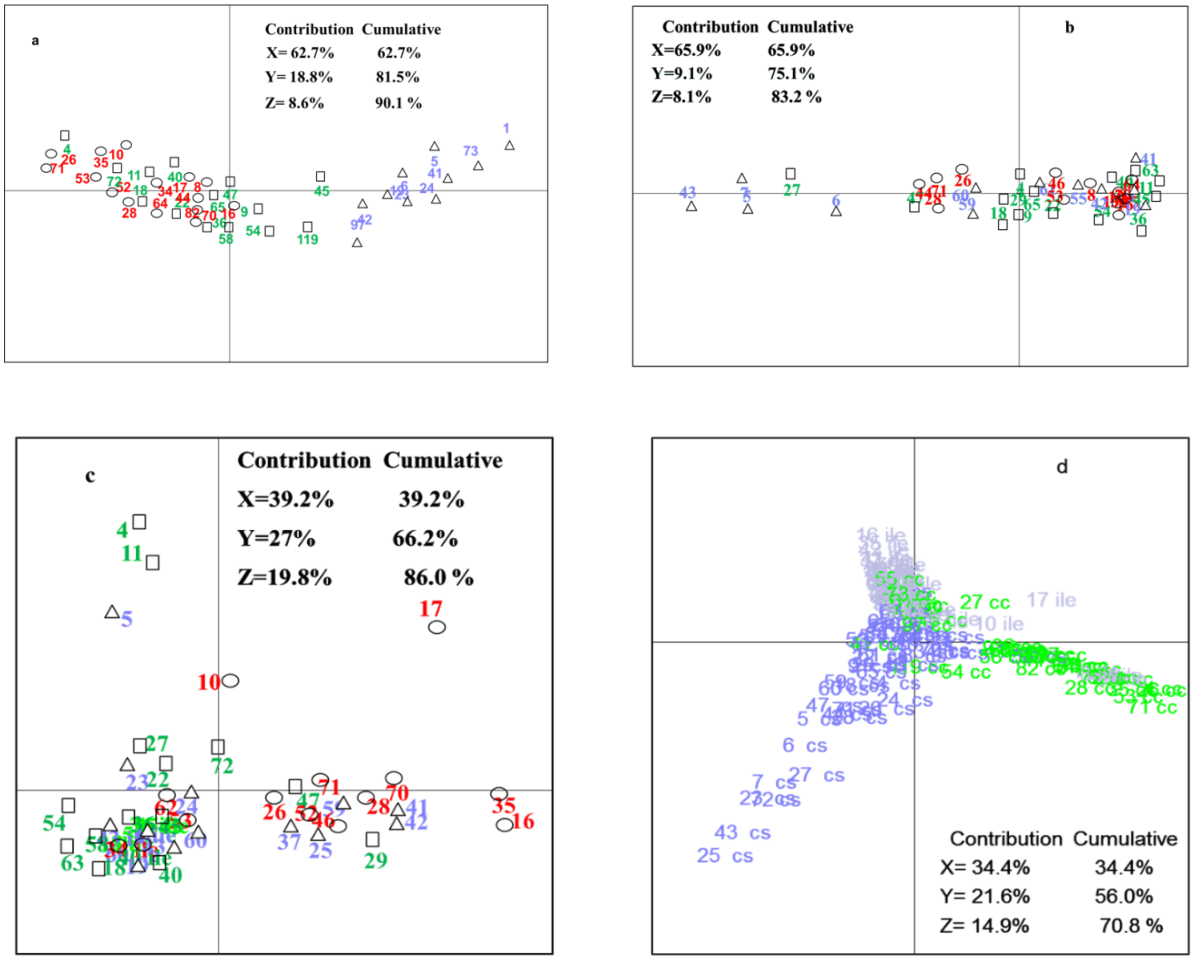


Figure 4.4. PCA analysis of broiler chicken gut microbial communities from (a) crop content (b) crop mucosa (c) ileum identified by diets; pea (triangles), wheat (squares), and corn (circles) while panel (d) represents segregation of crop content (green) , crop mucosa (blue) and ileum (grey) samples. Here, T-RFLP data over 1 % relative abundance threshold was considered for analysis by PCA.

4.4.3 Quantification of Selected Predominant Bacterial Groups and Species

The qPCR analysis of crop contents (Table 4.5) revealed that broilers fed corn- or pea- based diets had higher density of total bacteria and *Lactobacillus* spp. in contrast to birds fed wheat. Moreover, the increase in total bacteria and *Lactobacillus* spp. in crop contents of birds fed corn- or pea- based diets could be partly accounted for by increased abundance of *L. johnsonii* and *L. salivarius* in comparison to their wheat-fed counterparts ($P<0.05$). Indeed, the density of *L. johnsonii* in crop contents increased by meal-feeding versus *ad-lib* feeding only when pea-based diets were fed and in agreement with the clustering profiles (Figure 4.5).

For crop mucosa (Table 4.6) and ileum (Table 4.7), a few three-way interactions were recorded in the current study, although these were not evaluated due to a low number of observations. Only, two-way interactions observed between main factors were examined further for both broiler chicken GIT locations. For crop mucosa, total bacteria, *Lactobacillus* spp., *L. crispatus* and *L. reutri* density decreased when coarse diets were fed at *ad-lib*, however, the density of these bacteria did not change in response to grinding when fed as three meals per day (Figure 4.6). The crop mucosa *L. johnsonii*, *L. gallinarium and L. salivarius* density responded differently to meal feeding depending on the major feed ingredient (Figure 4.7). Meal feeding significantly increased the crop mucosa density of *L. johnsonii*, and *L. gallinarium* only when wheat-based diets were fed whereas *L. salivarius* density was decreased with meal feeding compared to *ad-lib* only when pea diets were fed (Figure 4.7).

Consistent with observations in crop mucosa, *ad-lib* feeding of wheat-based diets decreased *L. salivarius* density in ileum compared to corn- and pea- based diets independent of feeding frequency (Figure 4.8). Likewise, to crop mucosa (Figure 4.9), *ad-libitum* feeding of finely ground diets increased ileal total bacteria, *Bifidobacterium* and *L. crispatus* density whereas *Streptococcus* and *Lactobacillus spp.* density increased when coarse diets were meal-fed three times per day. Coherently, meal feeding also significantly increased *L. johnsonii*, *L. gallinarium* and *L. reutri* density in contrast to *ad-lib* feeding (Figure 4.9). Moreover, feeding corn based diets increased ileal *L. johnsonii* density while

feeding pea decreased density of both *L. gallinarium* and *L. johnsonii* versus corn and wheat based diets (table 4.7).

Table 4.5. Mean copy number (log_{10} gene copies /g of content) of selected bacterial groups and species in crop contents collected from 36 d old broiler chickens fed diets based on corn, wheat or pea at *ad-lib* or meal-fed frequency grounded to fine or coarser sizes (n=48).

Target bacteria	Dietary ingredient (D)			Feeding Frequency (F)		Screen Size (S)		Interactions (P-value)[1]				Pooled SEM
	Corn	Wheat	Pea	*Ad-lib*	Meal- fed	Fine	Coarse	D X F	S X F	D X S	D X F X S	
Total bacteria	10.40[a]	9.94[b]	10.46[a]	10.33	10.21	10.33	10.21	0.70	0.42	0.97	0.07	0.171
Total *Lactobacillus*	9.97[a]	8.80 [b]	10.23[a]	9.85	9.51	9.66	9.70	0.85	0.22	0.75	0.19	0.092
L. crispatus	8.67	8.21	8.84	8.60	8.54	8.52	8.63	0.85	0.25	0.69	0.40	0.137
L. johnsonii	8.67	7.27	8.21	7.65	8.45	7.73	8.37	0.01	0.85	0.35	0.35	0.187
L. salivarius	9.30[a]	8.14[b]	9.28[a]	9.03	8.78	8.85	8.96	0.53	0.63	0.59	0.08	0.179
L. gallinarium	7.39	7.50	7.45	7.25	7.64	7.38	7.51	0.80	0.55	0.78	0.15	0.135
L. reuteri	8.84	8.49	8.83	8.71	8.73	8.62	8.81	0.61	0.47	0.96	0.75	0.160

SEM-Standard error of the mean.

[1]D= dietary ingredient, F=feeding frequency, S=screen size.

[a,b]Means in same column with different letters are different at P <0.05.

Table 4.6. Mean copy number (log 10 copies of target gene/g of mucosa) of selected bacterial groups and species in crop mucosa collected from 36 d old broiler chickens fed diets based on corn, wheat or pea at *ad-lib* or meal-fed frequency grounded to fine or coarser sizes (n=48).

Target bacteria	Dietary ingredient (D)			Feeding Frequency (F)		Screen Size (S)		Interactions (P-value) [1]				Pooled SEM
	Corn	Wheat	Pea	*Ad-lib*	Meal- fed	Fine	Coarse	D X F	S X F	D X S	D X F X S	
Total bacteria	8.85	8.78	9.26	9.02	8.91	9.01	8.91	0.10	0.03	0.17	0.41	0.111
Total *Lactobacillus*	7.91	7.61	8.45	7.92	8.05	8.16	7.82	0.07	0.03	0.59	0.23	0.176
L. crispatus	6.59	6.76	7.05	6.63	6.98	6.98	6.63	0.06	0.01	0.22	0.05	0.118
L. johnsonii	5.28	5.48	5.44	5.03	5.76	5.50	5.30	0.02	0.06	0.44	0.66	0.120
L. salivarius	8.04	7.62	8.43	8.03	8.04	8.17	7.88	0.05	0.18	0.45	0.40	0.142
L. gallinarium	5.27	6.14	5.86	5.42	6.09	5.83	5.68	0.03	0.39	0.82	0.24	0.121
L. reutri	7.09	7.31	7.32	6.91	7.57	7.32	7.16	0.14	0.01	0.17	0.81	0.121

SEM-Standard error of the mean.

[1]D=dietary ingredient, F=feeding frequency, S=screen size.

[a,b]Means in same column with different letters are different at P <0.05.

Table 4.7. Mean copy number (log 10 copies of target gene/g of content) of selected bacterial groups and species in ileum content collected from 36 d old broiler chickens fed diets based on corn, wheat or pea at *ad-lib* or meal-fed frequency grounded to fine or coarser sizes (n=48).

Target bacteria	Dietary ingredient (D)			Feeding Frequency (F)		Screen Size (S)		Interactions (P-value) [1]				Pooled SEM
	Corn	Wheat	Pea	*Ad-lib*	Meal- fed	Fine	Coarse	D X F	S X F	D X S	D X F X S	
Total bacteria	9.62	9.11	9.24	9.37	9.27	9.35	9.29	0.23	0.03	0.63	0.04	0.104
Streptococcus	9. 41	8.71	9.20	8.90	9.32	9.03	9.17	0.053	0.01	0.93	0.03	0.110
Bifidobacterium	6.21	6.42	6.55	6.15	6.63	6.55	6.23	0.61	0.03	0.85	0.30	0.098
Total *Lactobacillus*	9.75	9.41	9.44	9.44	9.63	9.54	9.53	0.18	0.04	0.84	0.12	0.118
L. crispatus	7.17	6.59	6.12	6.35	6.90	6.74	6.51	0.08	0.01	0.11	0.12	0.140
L. johnsonii	7.49[a]	6.77[b]	6.54[b]	6.23[b]	7.65[a]	6.93	6.95	0.49	0.08	0.22	0.13	0.162
L. salivarius	8.84	7.94	8.17	8.21	8.43	8.21	8.43	0.03	0.42	0.94	0.18	0.127
L. gallinarium	6.50[ab]	6.95[a]	6.05[b]	6.19[b]	6.81[a]	6.44	6.56	0.12	0.74	0.63	0.09	0.137
L. reutri	7.62	7.47	7.09	6.99[b]	7.79[a]	7.49	7.3	0.32	0.67	0.48	0.60	0.137

SEM-Standard error of the mean.

[1]D=dietary ingredient, F=feeding frequency, S=screen size.

[a,b]Means in same column with different letters are different at P <0.05.

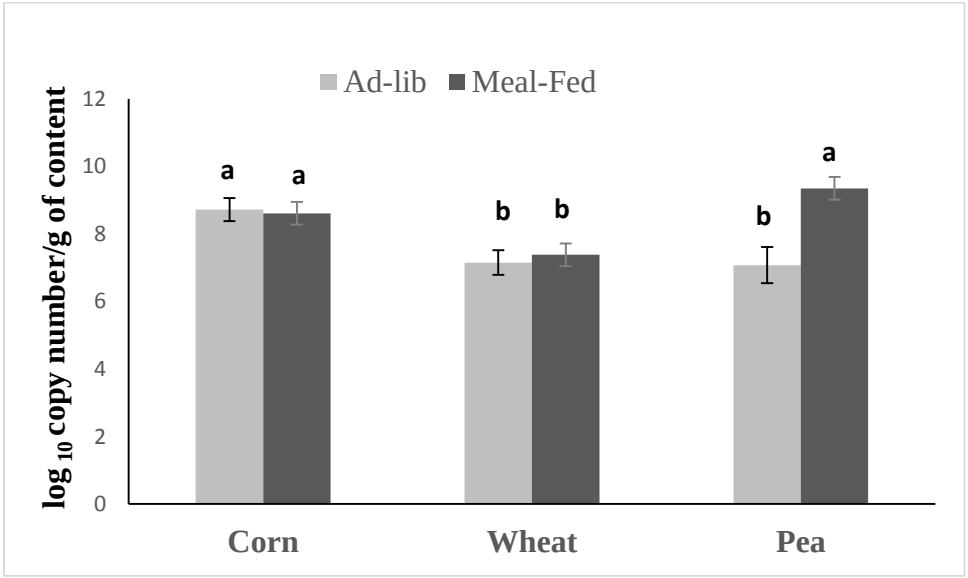


Figure 4.5. Mean number of copies of *L. johnsonii* (log_{10} copies of cpn60 gene/g of content) in crop content collected from 36 d old broiler chicken (n=48) fed corn, wheat or pea based diets at *ad-lib* versus meal fed (3X); error bars indicate standard error.

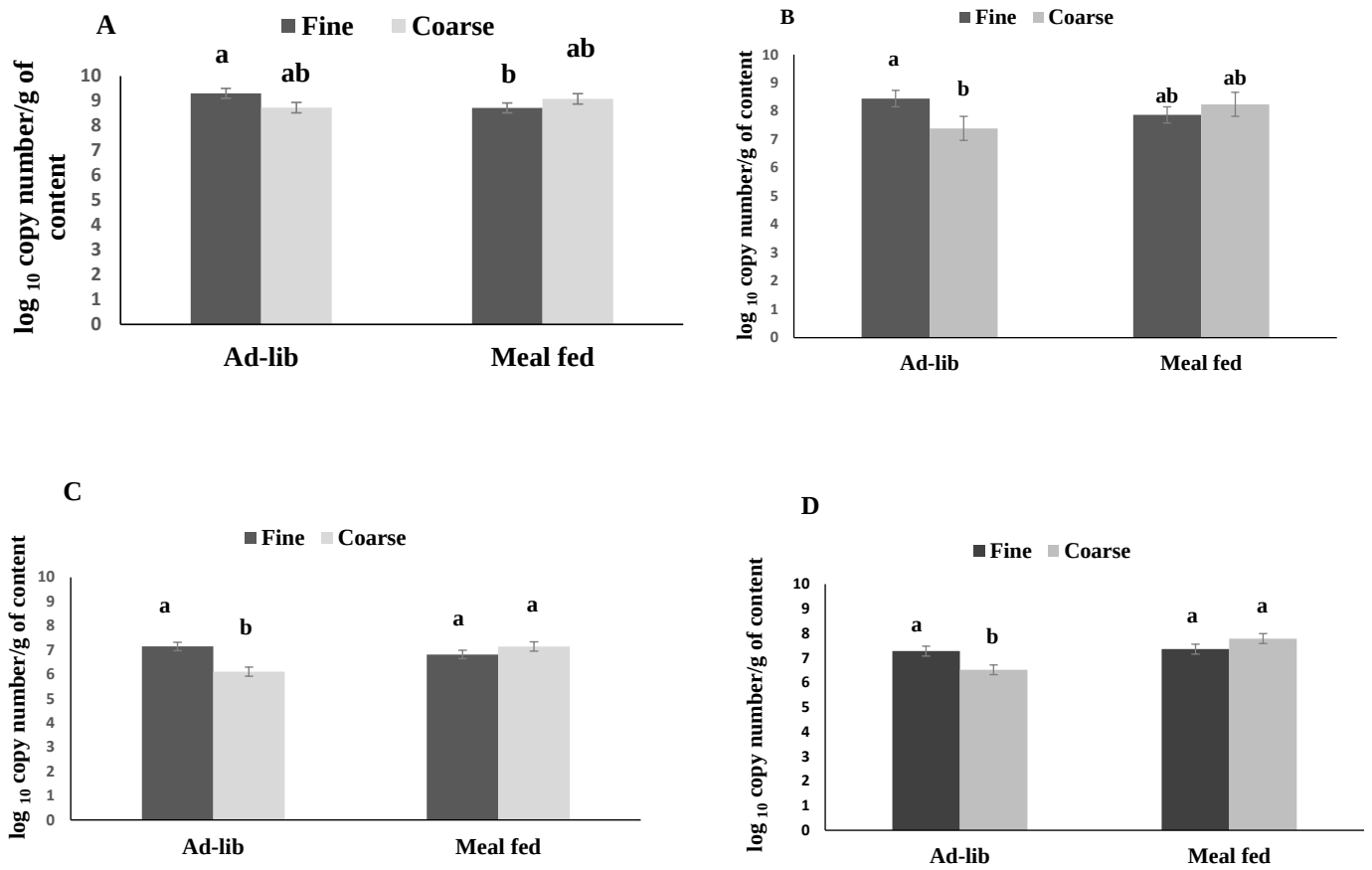
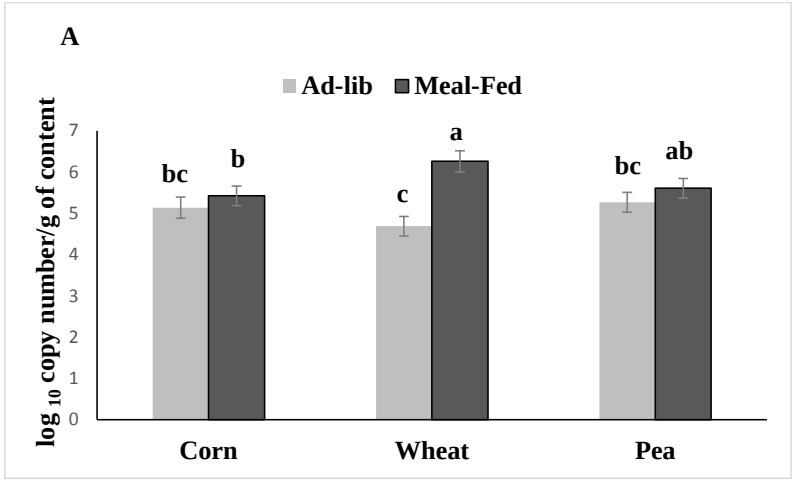
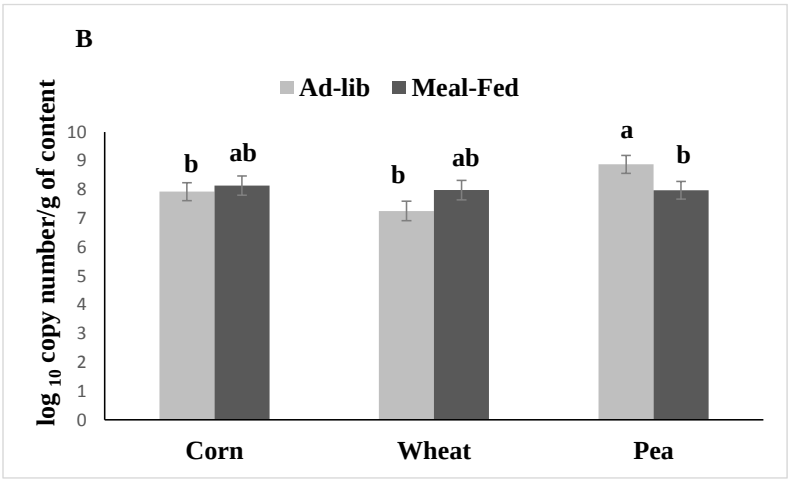


Figure 4.6. Mean number of copies (log_{10} copies of 16S rRNA gene/g of content) of target bacteria (A. total bacteria; B. *Lactobacillus*; C. *L. crispatus* (cpn60); D. *L. reutri*) in crop mucosa collected from 36 d old broiler chicken fed *ad-lib* or meal fed (3X) with fine or coarse grounded diets (n=48); error bars indicate standard error.





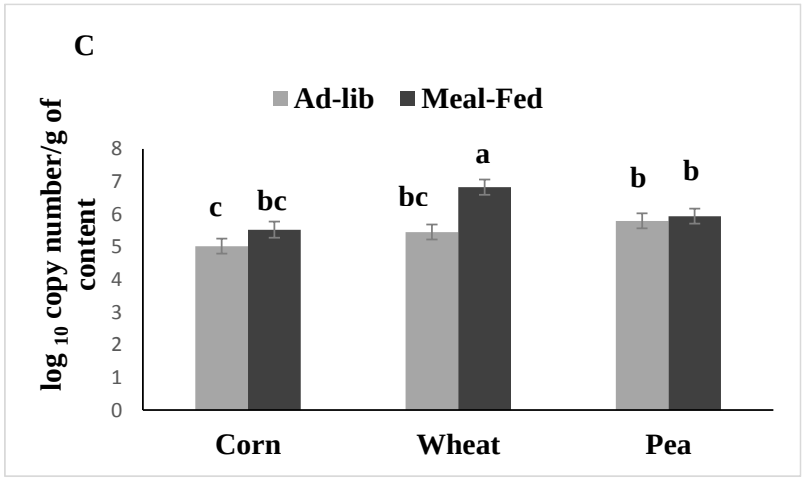


Figure 4.7. Mean number of copies (log_{10} copies of 16S rRNA gene/g of content) of target bacteria (A. *L. johnsonii* (cpn-60); B. *L. salivarius*; C. *L. gallinarum*) in crop mucosa collected from 36 d old broiler chicken fed corn, wheat or pea based diets at *ad-lib* or meal fed (3X) rate (n=48); error bars indicate standard error.

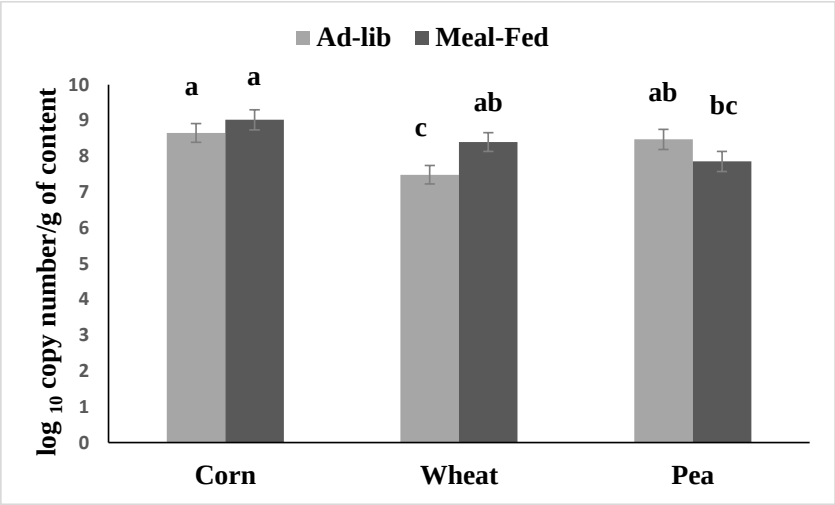
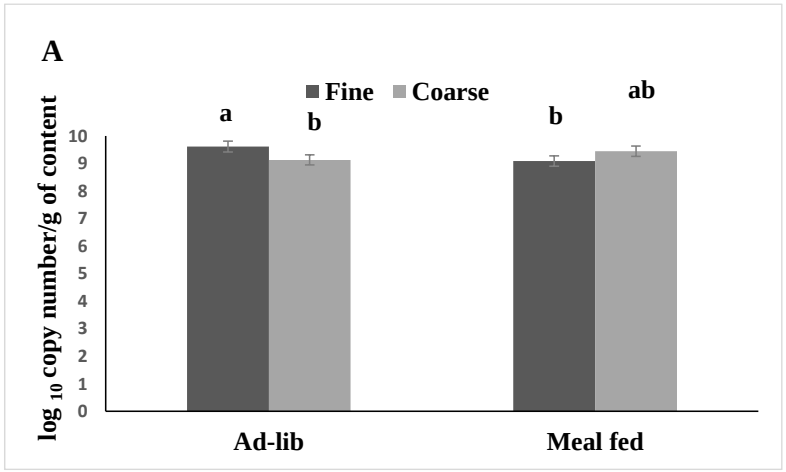
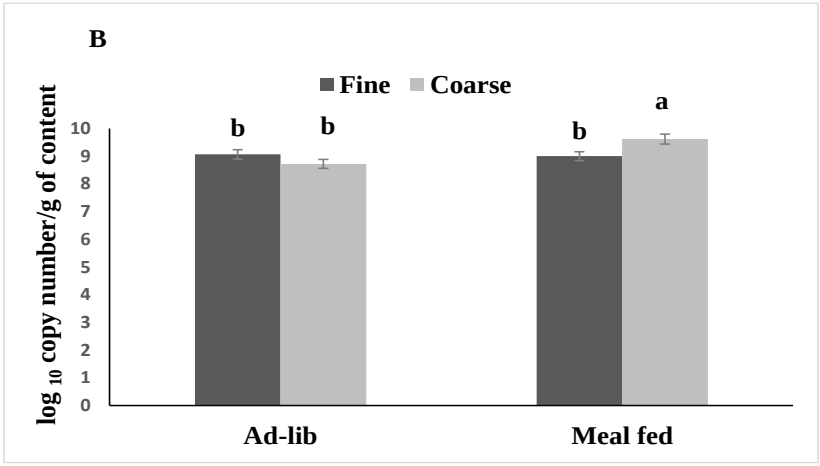
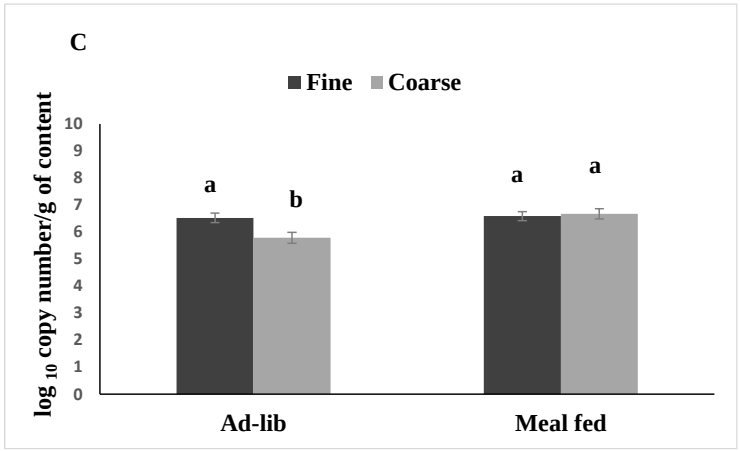


Figure 4.8. Mean number of copies (log_{10} copies of 16S rRNA gene/g of content) of *L. salivarius* in ileum content collected from 36 d old broiler chicken fed corn, wheat or pea based diets at *ad-lib* or meal fed (3X) rate (n=48); error bars indicate standard error.


A
Fine
Coarse
log 10 copy number/g of content
a
b
b
ab
Ad-lib
Meal fed


B
Fine
Coarse
log 10 copy number/g of content
b
b
b
a
Ad-lib
Meal fed


C
Fine
Coarse
log 10 copy number/g of content
a
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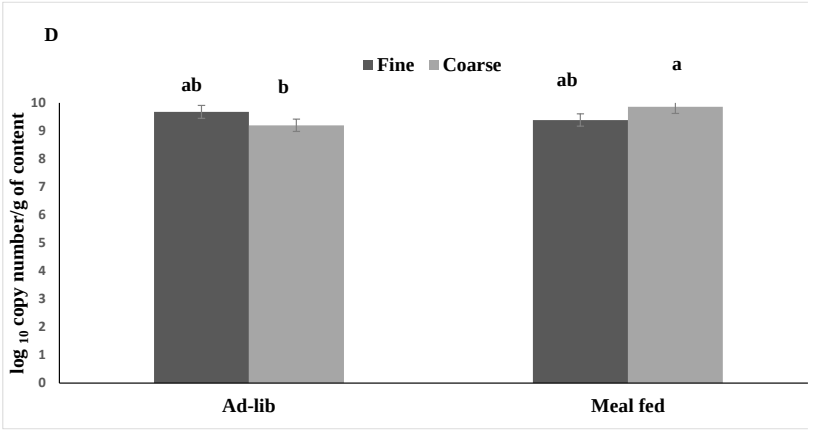
a
Ad-lib
Meal fed


D
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Meal fed

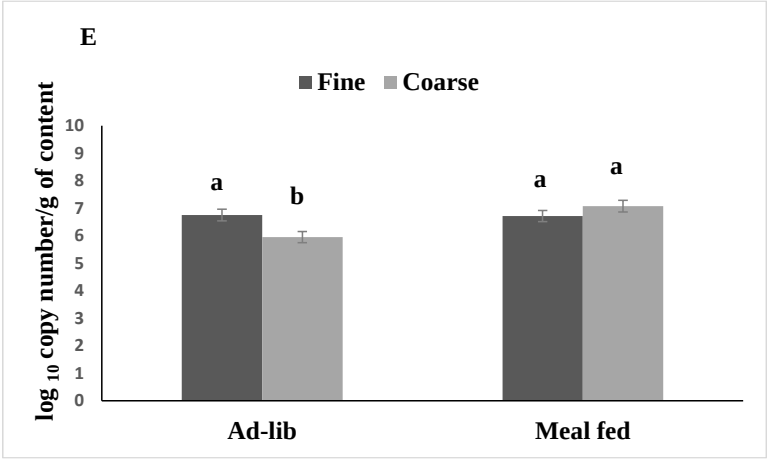

Figure 4.9. Mean number of copies (log_{10} copies of 16S rRNA gene/g of content) of target bacteria (A. total bacteria; B. *Streptococcus* group; C. *Bifidobacterium* group; D. *Lactobacillus*; E. *L. crispatus* (cpn-60)) in ileum contents collected from 36 d old broiler chicken fed *ad-lib* or meal fed (3X) with fine or coarse grounded diets (n=48); error bars indicate standard error.

4.5 Discussion

The resident gastro-intestinal tract (GIT) microbiota in poultry has considerable potential to influence intestinal tract development, health and performance as well as transmission of foodborne pathogens to humans (Hinton et al., 2000a, Gong et al., 2008; Yegani and Korver, 2008; Roberts et al., 2015). Current modern poultry production practices might hinder development of a "desirable" gut microbial community, which can be affected tremendously by environmental hygiene, in-feed antibiotics, artificial egg hatching, incubation and rearing of chicks (Gong et al., 2008). Diet composition is one of the major contributing factors (Yegani and Korver, 2008; Teirlynck et al., 2009a,b; Chambers and Gong, 2011; Witzig et al., 2015) in broiler chicken GIT community development with further influences of feed processing and form also reported (Engberg et al., 2002; Torok et al., 2011). Moreover, broiler birds have also demonstrated tremendous capacity to regulate crop feed storage patterns when adapted to intermittent or meal feeding (Svihus, 2014). This extensive and extended storage of feed in crop (Duve et al., 2011) might affect crop microenvironment affecting GIT entry of pathogens like *Salmonella typhimurium* (Hinton et al., 2000a; Scanes and Pierzchala-Koziec, 2014). Moreover, intestinal resident bacteria may utilize readily available nutrients (starch, simple sugars peptides) to support their growth (Shakouri et al., 2009; Pan and Yu, 2014) and have been suggested to equally compete with host for ingested dietary substrates (Langlands et al., 2004). Despite the potential importance of the crop microenvironment and high bacteria abundance especially *Lactobacillus* spp., compared to distal GIT locations in poultry, limited information is available on microbial community structure for upper gastrointestinal tract locations in response to changes in bird management. The present study demonstrated corn and pea based diets and meal feeding (three times per day) promoted crop *Lactobacillus* spp. colonization compared to wheat based or *ad-lib* fed diets, which could have implications for broiler gut health.

In present study, birds demonstrated expected growth performance reaching a slightly lower final weight than specified by Aviagen (2007) for Ross 308 growers, (http://www.natchix.co.za/pdf/performance_objectives.pdf) consistent with the slightly

lower energy content of the diets compared to the recommendation. Birds consuming pea-based diets revealed numerically higher feed intake over the last week of the study despite nearly 10% lower body weight than for wheat or corn, although differences were not significant. In support, earlier work feeding higher amounts of pea (60 %) have also demonstrated higher intake (Brenes et al., 1989) and similar performance to control corn-soy-wheat diets. Similarly, Czerwiński et al. (2010) found pea inclusion (@150 g/kg diet) in wheat-corn-soyabean meal based diets significantly increased feed intake recorded over 8-35 d period, however, pea inclusion has no effect on final body weight. This could be due to difference in pea total starch content, type, ileal digestibility, higher caecal fermentation of resistant starch (Daveby et al., 1998; Weurding et al., 2001; Czerwiński et al. 2010) compared to cereal grain (corn and wheat). As starch is the major energy source in poultry diet and starch digestibility has positive correlation with ingredient apparent metabolisable energy (AME) values value (Wiseman, 2000) which can then affect host performance. Not surprisingly, birds fed *ad-lib* had significantly higher final body weights versus meal-fed birds as reported previously (Svihus et al., 2010). We were unable to statistical compare feed intake although our values were similar for ad libitum and meal-fed birds. In agreement, Svihus et al. (2010), reported similar feed intake for broiler birds fed meals 5X per d (compared with 3X per d here) compared to *ad-lib*. Moreover, previous work examining the effect of feeding a coarser diet in poultry have shown better performance in contrast to fine sized diets due to better developed gizzard ensuring improved grinding, well controlled flow of ingested feed and digestive juices (Amerah et al., 2007; Svihus et al., 2010; Qaisrani et al., 2015). We did not observe any effect of screen size on performance of birds although the number of birds employed in the study may have been inadequate to detect a significant effect.

As anticipated, offering a meal three times per day increased the relative size and quantity of digesta present in the crop in accordance to previous published reports (Svihus et al., 2010). Earlier reports have shown, meal-fed birds tremendously adapt their feeding behavior by extensive utilization of crop for food storage permitting the slow release of food between meals and increased intestinal retention (Duve et al., 2011; Svihus et al.,

2010, 2014). Feeding a coarser diets did not affect relative crop size but did increase crop digesta content. In contrast, Qaisrani et al. (2015) found that broiler chickens fed fine (190 μm) rapeseed based diets had increased relative empty crop weight and crop contents compared to birds fed coarse (368 μm) diets. These contradictions (crop size and content) could be due difference in their installed photoperiod (16L:8D), feed type (pellet versus mash) and feed particle size (368 μm-coarse). Increased crop content weight recorded in present work could be due to differences in water holding capacity of feed particle size, where coarse particles have shown to hold more water compared to fine (Choct, 1997). Coherent to crop content, no influence of dietary ingredients was recorded on crop pH in present study which agrees with a study by Nishii et al. (2016) revealing no influence of dietary ingredient (grounded corn, whole grain paddy rice and brown rice) on crop pH measured at d 28 and 42. However, our results were contradictory to Czerwiński et al. (2010) who reported a lower crop digesta pH (4.75) when birds were fed pea versus wheat-soya-maize-based diets. Contradictory to our expectation, meal feeding did not result in decrease in crop pH compared to *ad-lib* broiler chickens. However, this finding agrees with Svihus et al. (2013) who reported a lack of feeding frequency (*ad-lib* vs 5X meal fed) effect on crop pH. Lower crop pH for fine wheat based oat hull diets vs coarse structure diets has been reported by Svihus et al., (2013) which disagrees with our findings. We did observe an increase in density of total bacteria and *Lactobacillus* spp. in crop content for pea and corn treatment groups, which might be expected to be associated with a lower pH due to increased microbial activity (Durant et al., 1999). However, pH measurement may not be sufficiently sensitive to assess microbial activity and/or the rate of disappearance of acidic fermentation products. Although speculative and needs further investigation, one possible reason for lack of main effects (*ad-lib* - meal fed; fine – coarse) effect on crop pH could be the timing of sample collection relative to the last consumption of feed. Furthermore, since collection of crop content occurred one hour after the morning meal, there may have been insufficient time for fermentation to lower the crop pH (Cutler et al., 2005).

Here, the T-RFLP based crop content and mucosa associated microbial community analysis revealed that corn and wheat-based diets fed broiler chicken samples clustered

together segregating from pea diet samples, mainly consisting of meal fed birds. Recently, another group demonstrated clustering of the T-RFLP profile in 25 d old Ross 308 birds where crop (Witzig et al., 2015) samples clustered according to diet composition, in this case, monocalcium phosphate content. The present work is consistent with several others, showing diet composition is a prime factor in determination of microbiota composition in any GIT location including crop (Yegani and Krover, 2008; Torok et al., 2011; Witzig et al., 2015; Nishii et al., 2016). Difference identified here, in crop lumen and mucosa associated bacterial community by molecular profiling technique can be explained as we have hypothesized earlier that in upper gut available dietary nutrients may be more important than unavailable nutrients such as resistant starch or fibre which could have potentially affected bacterial abundance at this GIT location. It is well known that ingested dietary ingredients are also a source of nutrients for resident bacteria (Lan et al., 2005; Shakouri et al., 2009), differences in dietary ingredients such as starch content, granule type, structure, degree of crystallinity and GIT digestibility values might have led to the observed differences community and bacterial target abundance (Daveby et al., 1998; Weurding et al., 2001; Czerwiński et al., 2010; Zaefarian et al., 2015). As evident from the present study, nutrient content analysis of experimental diets where (Table 4.1) similar starch content of cereal grain (corn and wheat) and lower resistant starch content compared to pea can be associated well with T-RFLP results. Typically, dietary starch is the major host energy source (Weurding et al., 2001), however, this starch can also be used efficiently by intestinal bacteria for their own growth (Shakouri et al., 2009). On the other hand, resistant starch cannot be effectively utilized by either the host or its GIT bacteria and is considered resistant to microbial action in upper gut associated with short residency (Campbell and Bedford, 1992). Moreover, *Lactobacillus* species have been recognized as strictly fermentative bacteria utilizing several readily available substrates (carbohydrates, amino acids, peptides, fatty acid esters etc) and producing primarily lactic acid as an end product (Tannock, 2004; Scanes and Pierzchala-Koziec, 2014). Therefore, given the abundance of *Lactobacillus* spp. reported in crop (Fuller and Turvey, 1971; Scanes and Pierzchala-Koziec, 2014; Witzig et al., 2015; Saxena et al., 2016), it was expected in the

current work that the *Lactobacillus* population would be primarily influenced by the highly available nutrients (starch content – Corn > Wheat > Pea) of main dietary ingredient in each diet. Moreover, it has also been suggested that (Weurding et al., 2001) starch digestion occurred mainly in the broiler chicken upper small intestine and prominent differences among upper GIT starch digestibilities for various common poultry feed stuffs (corn, wheat and pea) have been recorded. This could explain our T-RFLP profiling result which were then confirmed further with qPCR enumeration.

In the current study, an array of predominate bacterial species previously identified in broiler chicken crop were chosen for quantification by qPCR (Guan et al., 2003; Hilmi et al., 2007; Rehman et al., 2007; Witzig et al., 2015) which revealed diets containing wheat reduced total bacterial density and counts of *L. salivarius* and *L. johnsonii* in crop content versus corn and pea diets. In agreement to qPCR analysis of our work, culture-based analysis of total crop bacteria and *Lactobacillus* species counts in 28 d old broiler chicken revealed similar values for pea (60 % of diet) and corn-soy-wheat control diets (Brenes et al., 1989). Brenes et al. (1989), using culture based methods also found similarity in lactobacilli counts in crop content taken from birds fed corn and pea-based diets. However, these findings with qPCR in the current study are in discordance with T-RFLP analysis which identified crop content microbiota from birds fed pea diets as dissimilar from birds fed corn or wheat diets. Since pea typically contains less starch and have a greater proportion of resistant starch compared to corn and wheat diets. Therefore, we anticipated total bacteria and *Lactobacillus* spp. density in pea based diets to be dominantly affected as starch is preferred substrate for lactobacilli (Tannock, 2004; Scanes and Pierzchala-Koziec, 2014). Nevertheless, wheat diets, were associated with lower bacterial counts assessed by qPCR which presumably could be due to higher total NSP content (Hetland et al., 2004). In agreement with our findings, Shakouri et al. (2006) conducted a broiler chicken experiment examining the effect of dietary inclusion of different NSPs and revealed NSP inclusion decreased lactic acid bacterial counts primarily in proximal gut locations, duodenum, jejunum and ileum. In contrast, using 3-4 week-old broiler chickens fed lupin supplemented diets containing high amounts of total NSP (360 g/kg lupin seed

meal), Rubio et al. (1998) reported a significant increase in crop lactobacilli counts compared to a control wheat-soyabean diet irrespective of age. The impact of soluble fibre in crops still remains ambiguous and need further studies. Compared to insoluble fibre content of dietary fibre mostly affect digesta transit time and can not be utlilized as efficiently by intestinal bacteria for fermentation, soluble fraction increase viscosity, digestive organ size, GIT secretions and anaerobic bacteria (Hetland et al., 2004; Choct, 1997). The use of a xylanase in wheat and pea based diets in the current study might have also affected surface availability for bacterial action which could further influence microbial abundance in the crop (Vahjen et al., 1998, Yang et al., 2009) assuming residency of content (Weurding et al., 2001) and pH permitted substantive activity.

Here, while investigating broiler chicken upper gut microbial community composition, T-RFLP profiling assay revealed pea diets were different, although on other hand, qPCR enumeration of selected bacterial targets suggested wheat was different. This could be due to the fact that T-RFLP is a semi-quantitative fingerprinting technique mainly predicting shifts in abundant microbial community members, however, unable to precisely discriminate or quantify bacteria species/group responsible for change (Ruiz et al., 2015). This technique is complementary with qPCR methodology, which is a highly quantitative technique capable of measuring abundance of specific selected target bacteria (Gong et al., 2008; Zoetendal et al., 2004) of a microbial community. Therefore, results indicated by both these techniques seems discordant, however, as they measure different targets, so, eventually they do not disagree.

In agreement to previous research findings, qPCR analysis here, revealed crop mucosal microbiota was found to be distinct from microbiota associated with contents (Fuller and Turvey, 1971; Gong et al., 2007; Malmuthuge et al., 2012). Mostly two-way interactions were recorded between diet and feeding frequency for examined bacterial species in crop mucosa. For instance, wheat diets increased density of *L. johnsonii*, and *L. gallinarium* only when meal fed while *ad-lib* feeding of pea diets increased *L. salivarius* in crop mucosa. All of this agrees with the suggestion that the mucosa-associated community may require a longer exposure time to adapt to diet composition compared to

luminal community which is transient in nature (Thompson et al., 2008). Furthermore, the influence of dietary carbohydrases on bacterial community composition has been suggested to be more noticeable in the mucosa-associated community rather than lumen (Vahjen et al., 1998), agreeing to present work. Vahjen et al. (1998) recorded, a positive correlation between NSP degrading enzyme and mucosa associated *Lactobacillus* population rather than lumen. Agreeing to current findings, xylanase supplemented pea and wheat diets supporting higher abundance of *Lactobacillus* species in crop mucosa. In support, Engberg et al. (2004) broiler work indicated xylanase supplementation of wheat diets did not influence lactic acid bacteria and *L. salivarius* counts in broiler chicken gizzard.

For ileum, T-RFLP profile analysis did not reveal any effect of dietary ingredients despite numerous reports that dietary composition is a major factor affecting ileal microbial composition (Rehman et al., 2007) including experiments where T-RFLP was employed as the assessment tool (Torok et al., 2008; Witzig et al., 2015). The qPCR enumeration of selected bacterial targets in ileum in the current experiment revealed some differences associated with diet. For example, corn diets promoted *L. johnsonii* whereas feeding wheat increased *L. gallinarium* density in contrast to pea-based diets which decreased density of both these species in ileum; possible consistent with low available starch. Weurding et al. (2001), reported approximately 90 % and 98 % of wheat starch is digested before the ileum and prior to posterior ileum, respectively. In contrast, pea have a total starch content of approximately 47 % (Czerwiński et al., 2010) and a digestion coefficient of 81 % at posterior ileum in broiler chickens (Weurding et al., 2001). Daveby et al. (1998) have further reported that this low apparent ileal digestibility of pea starch was due to high amylose to amylopectin ratio and abundance of C type of starch granules. Moreover, inclusion of NSP degrading enzyme addition to broiler diets may modify microbial fermentation of substrate in the ileum (Meng and Slominski, 2005).

Each location along the digestive tract has been reported to harbor distinct microbial communities as observed here and elsewhere (Lu et al., 2003; Rehman et al., 2007; Yegani and Korver, 2008, Malmuthuge et al., 2012; Stanley et al., 2014) due to

distinct environment and functional properties of that intestinal location. Nevertheless, communities in each location could influence the composition in neighboring environments. For example, in the present study, three times per meal versus *ad-lib* feeding revealed to increase abundance of *L. johnsonii* at all three investigated locations (crop content, crop mucosa and ileum contents) during this work. Coherently, besides being quite diverse, few identical bacterial targets have been identified between adjoining intestinal compartments (Wielen et al. 2002; Rehman et al., 2007).

We anticipated that three times per day meal feeding would have a prominent influence on the proximal gut *Lactobacillus* population based on earlier reports suggesting extended residency of crop ingesta (Durant et al., 1999; Svihus et al., 2010; Svihus, 2014). T-RFLP based cluster analysis of crop microbial community did reveal a distinct cluster however, only for meal fed birds given pea diets. Moreover, this effect was not observed for crop mucosa and ileal microbial community examined in this study by T-RFLP assay. This could be due to the suggestion that similar total small intestine (jejunum to posterior ileum) mean retention values for corn, wheat and pea (Weurding et al., 2001). Although, qPCR investigations did indicate an increase in *Lactobacillus* species especially *L. johnsonii* across crop content, mucosa and ileum, in comparison to *ad-lib* meal feeding of diets. This can be speculated due to extended broiler chicken intestinal residency (Svihus, 2014). However, processing of microbial community samples collected only at one time point shortly after meal might be another speculated reason for prominent meal feeding response observed in this study.

T-RFLP based microbial profiling of crop content, crop mucosa and ileum content did not identify community responses due to screen size. Using qPCR, *ad-lib* feeding of fine diets positively influenced density of total bacteria, *Lactobacillus* spp., *L. crispatus*, and *L. reutri* in crop mucosa and ileal *Bifidobacterium* spp. and *L. crispatus*. Furthermore, higher *L. crispatus* density was recorded in the crop mucosa and ileum contents associated with feeding of finely ground *ad-lib* diets supporting the suggestion that the crop microbiota may act as inoculum for lower gastrointestinal section (Fuller, 1973; Edelman et al., 2002; Wielen et al., 2002; Hilmi et al., 2007). Intestinal epithelial cells adherence

capabilities of bacterial species have been recognized as prime factor for intestinal colonization in broiler chicken (Fuller, 1973; Edelman et al., 2002). Moreover, compared to mucosa attached, lumen bacteria have been proposed to be transitory in nature, which might have detached from preceding intestinal sites (Fuller and Turvey, 1971; Savage, 1972; Fuller, 1973, 1978). Earlier, *in-vitro* adhesion specificity test along the chicken alimentary tract has also identified *L. crispatus* predominance at both sites; crop and intestinal epithelium niches, (Edelman et al., 2002) compared to *L. gasseri* and *L. reuteri*, due to strong adherence capabilities.

Furthermore, based on results of this present work, it can be speculated that compared to coarse, finely ground diets might provide greater surface area for digestive or microbial enzymes, less abrasive action, epithelial desquamation and faster solubilization (Cheng and Hironaka 1973; Mccowan et al., 1980; Mikkelsen et al., 2004; Amerah et al., 2007) affecting bacterial density especially associated with a faster rate of passage when fed *ad-lib* versus meal feeding. An examination of pig stomach contents revealed rheological differences between fine and coarse diets, after mixing with water, fine diets separates into to watery phase and precipitate while coarse diet form thick viscous fluid (Mikkelsen et al., 2004). In contrast to our hypothesis, others have reported increased (Mikkelsen et al., 2004) or no effect of grind size on intestinal microbial abundance (Engberg et al., 2002). For instance, Engberg et al. (2002) reported no influence of grind size (fine versus coarse mash wheat based diets) on abundance of total anaerobic, lactic acid bacteria, *L. salivarius* and other lactobacilli investigated in broiler chicken gizzard to rectum. This could be due to differences in bird type (Ross 208 versus 308), age of analysis (42 d versus 36 d old) and method of analysis (culture versus molecular). Several counter-intuitive studies have generally assumed that feeding coarser diets positively influence gizzard development, slow digesta passage, thicken digesta consistency, decrease pH (Ruhnke et al., 2015, Qaisrani et al., 2015; Nishii et al., 2016), increase gastric secretions (Engberg et al., 2002) and microbial fermentation (Mikkelsen et al., 2004). However, both *in-vitro* and *in-vivo* broiler chicken experiment has suggested finely ground pea diets improved rate and extent of starch digestion along with broiler chicken performance

examined over 14-22 d (Ebsim, 2013) due to better access to starch granules. Due to prevalent discrepancy regarding influence of feed particle size on intestinal microbial communities, more work is required in future directly correlating diet dependent microbial ecology changes in the different broiler chicken intestine locations along with a pathogen (*Salmonella* or *E. coli*) challenge.

4.6 Conclusions

The current broiler chicken study has demonstrated corn and pea feeding supported *Lactobacillus* species in proximal gut compared to wheat based diets. In broiler chicken foregut, easily digestible substrates (e.g. available starch – Corn > Wheat > Pea) may be serve as an important substrate for microbiota, however, unavailable substrates such as fibre (Wheat > Pea > Corn) and resistant starch (Pea > Corn > Wheat) may also influence intestinal microbial community composition. Meal feeding increased *Lactobacillus* species abundance, initiating at crop level and persisting through small intestine. Diet composition, processing and feeding strategies may interact to enhance colonization by putatively beneficial bacteria such as Lactobacillus species and should be considered among management strategies to support gut health.

5.0 GENERAL DISCUSSION

Photoperiod, duration of light exposure per day (L), is among one of the common management tools implemented in commercial poultry production to alter growth (Olanrewaju et al., 2006; Schwean-Lardner et al., 2012, 2016) and feeding patterns of exposed birds (Buyse et al., 1993; Schwean-Lardner et al., 2014). Several photoperiod regimes are practiced across world; depending on market demand. Traditionally, extended photoperiods (e.g. 24L or 23L) were chosen to achieve maximum gains, as birds eat mostly during light phase (Olanrewaju et al., 2006; Schwean-Lardner et al., 2012a, 2016). However, currently, there has been increased interest in broiler chicken health, and welfare which has driven a re-examination of lighting programs (Durant et al., 1999; Ricke, 2003; Abbas et al., 2008; Schwean-Lardner et al., 2012a,b, 2013, 2014, 2016). Hypothetically raising birds under 23L/24L will yield the heaviest birds, however, in contrast to this assumption, different photoperiod regimes allowing more darkness exposure (e.g. 17L, 20L) have demonstrated identical final body weight, higher feed efficiency, better welfare, immune function and livability (Abbas et al., 2008; Schwean-Lardner et al., 2012a,b, 2013, 2014, 2016).

Darkness exposure has been suggested to have a positive effect on the avian immune system acting mainly through darkness-induced melatonin secretion from the pineal gland (Olanrewaju et al., 2006; Walton et al., 2011; Schwean-Lardner et al., 2014, 2016). Immune system health is a key factor in broiler chicken survival, growth and protection from pathogens, including those of zoonotic importance (Kogut, 2009). Typically, the immune system of the chick is still immature and naïve at the time of placement in traditional commercial conditions (Moore and Siopes, 2002; Olanrewaju et al., 2006; Kogut, 2009). Furthermore, it is under constant threat due to presence of several infectious and non-infectious stressors, which have potential to adversely affect immune

health status (Kirby and Froman, 1991; Kliger et al., 2000; Kogut, 2009). Hence, careful selection of a photoperiod regimen during the initial stages of broiler chicken life, that would promote better immune capabilities, might help birds to attain desired weight along with better survivability and competent immune response capabilities.

Initially, four different experiments were conducted, to investigate effect of photoperiod, ranging from 13L to 23L, on immune status in broilers chickens, imitating commercial poultry conditions by assessing several innate and acquired immune responses. It was indicated in this study that constant exposure of broiler chickens to 23L reduced organ weights, increased H:L ratios and infectious mortalities. However, inconsistencies were recorded for these immune response parameters across different experiments of this study, which agrees to previous studies (Onbaşılar et al., 2007; Wang et al., 2008; El Sabry et al., 2015, Yang et al., 2015b; Vermette et al., 2016). Therefore, it was difficult to draw conclusive interpretation after reviewing immune parameter results only.

The chronic LPS challenge model used here, has been employed to examine the effect of inflammation on growth performance, and indeed, growth performance was reduced here agreeing to earlier broiler experiments (Korver et al., 1998; Webel et al., 1998). There are also studies reported in Siberian hamsters (Prendergast et al., 2003) and broiler chickens (Gehad et al., 2008) investigating the effect of photoperiod on LPS induced inflammatory response indicating darkness mitigates the challenge response by diminishing immune response generation and thereby improving host survival. A novel attempt was made here, however, the lack of differential growth and immune response to LPS challenge suggests that the inflammatory response was not different under different photoperiods contrary to our hypothesis.

Furthermore, chickens have naturally low intensity of heterophil phagocytosis, compared to other species, with peak value observed during middle of dark phase (Rodriguez et al., 1999; Hriscu, 2004; Papp and Smits, 2007). It would therefore be desirable to conduct the heterophil function assay by blood sample collection over several time points spread across a day and night/dark period in broiler chickens of different ages,

to give a complete overview of the heterophil phagocytosis diurnal profile with age and photoperiod regime. Moreover, it is difficult to determine the particular reason for observed inconsistencies of immune parameters. It might be possible that the effect of photoperiod if present might be subtle, evading detection, but may exaggerate in a stress or disease condition. Still controversial, one other speculated reason for recorded immune response inconsistencies in this study could be due to intensive genetic selection of modern broiler chicken for faster growth rate (Qureshi and Havenstein, 1994; Cheema et al., 2003) and diminishing immune response generation capabilities. Moreover, adding to the difficulty is the dilemma whether heightened immune responses are indicative of better immune health or in contrast represents an ongoing stressed state (Smith and Hunt, 2004). In the future, it would be interesting to incorporate hormonal (melatonin or corticosteroid) assessment along with other established immune indicators at various time points during the photo and scoto - phases of a day, which would provide more strength and allow better interpretation of data. A follow up concurrent infectious challenge study with photoperiod would be interesting.

In addition to a direct effect of photoperiod duration on immune function mediated by melatonin, extended darkness exposure may have another potential beneficial effect on bird health associated with increased crop utilization mediated by altered feeding behavior (Buyse et al., 1993; Duve et al., 2011). This has been identified as a potential mechanism, preventing pathogen colonization in crop and whole GIT (Fuller, 1973; Hilmi et al., 2007; Scanes and Pierzchala-Koziec, 2014; Classen et al., 2016) due to pathogen exclusion by a more abundant crop microbiota and possible a distal gut seeding effect. Furthermore, this could be one of the reasons for recorded reduced infectious mortalities assessed for broiler chickens health in initial photoperiod and immune function experiments. Similar to photoperiod, the dietary amino acids constituting the most important and expensive ingredients of chicken diets have also been shown to affect performance parameters, feed intake, immune function and intestinal microbiota (Corzo et al., 2005; Dahiya et al., 2005; Lan et al., 2005; Brickett et al., 2007; Li et al., 2007; Dai et al., 2011; Lilly et al., 2011). Therefore, in a second experiment, photoperiod and dietary factors were examined for

performance, breast yield and crop microbial ecology in broiler chickens. The 16S rRNA gene based T-RFLP molecular fingerprinting method was implemented to investigate crop microbial community shifts due to photoperiod and dietary manipulation. The T-RFLP method was chosen due to its rapid, reliable and cost-effective nature; it was demonstrated to be a viable microbiota analysis tool. Although next generation deep sequencing is the current preferred microbial analysis method, at the time of these studies deep sequencing methodologies were prohibitively expensive. Recently, when T-RFLP was used concurrently with next generation techniques, authors reported that T-RFLP competently demonstrated identical results regarding abundant bacterial species (Witzig et al., 2015) supporting its continued use as a profiling tool.

The second experiment results mostly agree with earlier research and revealed a positive effect of photoperiod effect on growth performance and meat yield parameters (Hassanzadeh et al., 2012; Schwean-Lardner et al., 2012a). As expected, increased photoperiod (≥18L) decreased crop size (Buyse et al., 1993), increased crop pH (Cutler et al., 2005), and decreased the relative abundance of TRFs representing *Lactobacillus* (McEwan et al., 2005; Bailey et al., 2010; Walton et al., 2011). Moreover, a significant negative correlation was observed for 177 bp TRF abundance in the crop, representing *Lactobacillus* spp. and final body weight of broiler chickens similar to a study by de Lange and Wijtten (2010). Interestingly, the qPCR enumeration revealed that 23L photoperiod exposure decreased total bacteria, *Lactobacillus* group bacteria and *L. gallinarum* counts in crop content, compared to 13L exposed birds. This experiment provided novel in-depth quantification of the crop microbial ecology community of broiler chickens raised under different photoperiod and feeding conditions. Furthermore, based on crop microbial ecology results of this experiment and previous published literature (Durant et al., 1999; Hinton et al., 2000a; Ricke, 2003; Scanes and Pierzchala-Koziec, 2014) it can be suggested that darkness exposure could have a beneficial effect on whole gut health by increased crop *Lactobacillus* species abundance and reduced pH. However, due to semi-quantitative nature of T-RFLP technique (Ruiz et al., 2015), it was difficult to identify and quantity all bacterial groups or species which could have led to recorded differences in the crop

microbiota profiles in this work. In the future, implementation of high through put next gen sequencing techniques which are currently becoming more advanced in technology, cost effective, less laborious and could provide reliable in-depth information about GIT microbial community members rapidly for large set of samples, would also add the much required strength to existing data (Park et al., 2013).

Moreover, data is still lacking for nutritional requirement of normal inhabitants of the crop. For instance, it was difficult to clearly identify reason in this work, why *L. gallinarum* was promoted in broiler chicken crop raised under 13L. To the author's knowledge no study is present which identifies single or multiple growth requirements which can promote specifically this organism in broiler chicken gastro-intestinal tract. Moreover, crop samples were collected at one time point shortly after lights came on and after birds consumed a morning meal. It would be interesting to understand the effect of residency time in the crop on microbial composition to better understand and contrast ecology in the crop over an entire 24 h cycle. In future, metabolomics, quantitative and qualitative assessment of host metabolites in response to dietary interventions (Park et al., 2013), if conducted would be an added asset in data interpretation and conclusions.

In order to further examine effect of common dietary ingredients and strategies prevalent in the poultry industry, finally a broiler chicken trial was conducted to investigate their role on the crop microbial ecology that might support gut health. During this experiment different dietary ingredients (corn, wheat, pea), hammer mill screen size (coarse or fine) and feeding frequency (*ad-lib* or three times per day meal-feeding), were implemented to study effect on proximal GIT section and ileum microbial community by 16S rRNA gene based molecular profiling techniques. As anticipated, crop content and crop mucosa T-RFLP based analysis revealed segregation based on the primary dietary ingredient. Moreover, in agreement to earlier findings (Gong et al., 2002; Malmuthuge et al., 2012), irrespective of dietary strategy, radical differences were recorded for crop content and mucosa associated microbial community, assessed here by T-RFLP analysis and qPCR assay. In addition, PCA analysis of T-RFLP data revealed segregation of crop content, crop mucosa associated and ileal microbiota community, which agrees with earlier

findings indicating each poultry GIT segment has its own distinct and diverse microbial profile (Rehman et al., 2007; Yegani and Korver, 2008; Wilkinson et al., 2016). Unfortunately, mainly interactions were noticed between main factors, diet and feeding frequency or feeding frequency and screen size, for crop mucosa and ileum enumerated bacterial species, of this study. Therefore, it was not possible to correlate one single factor contributing significantly to microbiota member shifts. Furthermore, none of the dietary strategy adopted in this study influenced ileum T-RFLP profile clustering, despite previous findings that dietary composition is well recognized as major factor affecting ileal microbial composition (Rehman et al., 2007). This could be due to reported significant starch digestion differences of cereal grains (wheat and corn) of which > 90 % is absorbed prior to theposterior ileum compared to the apparent low ileal digestibility of pea starch (Daveby et al., 1998; Weurding et al., 2001; Czerwiński et al., 2010). Another speculated reason could be the NSP degrading enzyme addition to these diets which might have influenced ileum microbial fermentation soluble and insoluble NSP substrates (Meng and Slominski, 2005), potentially further affecting inhabitant microbiota (Montagne et al., 2003).

Furthermore, qPCR enumeration revealed wheat-based diets reduced *L. johnsonii* and *L. salivarius* density in the crop compared to corn and pea based diets. Moreover, compared to *ad-lib* fed broilers chickens, three times per day meal feeding increased *Lactobacillus* member species abundance at all three examined locations, crop content (*L. johnsonii*), crop mucosa and ileum (*L. johnsonii*, *L. gallinarum*, *L. reutri*). These findings are consistent with increased residency time of feed in crop associated with meal feeding and the resulting influence on substrate availability affecting bacterial species abundance (Buyse and Decuypere, 2003; Shakouri et al., 2009; Svihus, 2014). Coherently, *L. crispatus* density increased both in crop mucosa and ileum after *ad-lib* feeding of finely ground diets (Engberg et al., 2002; Péron et al., 2005) possibly could be due to better access to nutrients and faster nutritent solublization. Interestingly, these two unique observations here, meal feeding and *ad-lib* feeding of fine diets affecting abundance of similar *Lactobacillus* species (*L. johnsonii, L. gallinarum, L. reutri, L. crispatus*) species in crop mucosa and

distal gut (ileum) were in line with earlier reports. Those earlier findings suggested a role of crop microbiota mostly for autochthonous crop mucosa versus allochthonous lumen bacteria, as inoculum for ingested food and affecting microbial composition in the remainder of the gut (Fuller and Turvey, 1971; Savage, 1972; Fuller, 1973, 1978; Edelman et al., 2002; Wielen et al., 2002; Hilmi et al., 2007). In the future, additional work in this context, investigating several bacterial targets along whole broiler chicken GIT would be interesting. Weekly performance and feed intake observation simultaneous to short chain fatty acid analysis of the crop contents would be advantageous. Moreover, in today's era of new methodologies like next generation sequencing, metabolomics, *in-silico* prediction of metabolic product and its functions, if conducted in future, will aid in more in-depth analysis of composition and function of microbial communities. Based on results of this study, it can be concluded that easily digestible substrates (e.g. available starch, amino acids) may serve as an important substrate for proximal broiler chicken intestinal microbiota microbiota, however, unavailable substrates such as fibre and resistant starch might also influence intestinal microbial community composition particularly crop *Lactobacillus* species colonization can be promoted by diet composition, processing and feeding strategies.

To conclude, the primary objective of the first study (Chapter 2) examining effect of photoperiod on the immune status and health of broiler chickens assessed based on several innate and acquired immune parameters was partially accomplished. As consistencies were recorded for examined immune parameters during this study, however, if combined with earlier findings this study does indicate darkness exposure could positively influence immune function and health of broiler chickens (Abbas et al; 2008; Kliger et al., 2000; Schwean-Lardner et al., 2016).

Regarding the second objective, aimed at investigating effect of photoperiod and amino acid levels on growth performance, meat yield, and crop microbial ecology by 16S rRNA gene based profiling techniques (T-RFLP and qPCR) was accomplished (Chapter 3). The broiler chicken growth performance and breast meat yield results indicated improvement with both photoperiod and dietary amino levels. However, compared to

continuous (23L), reduced photoperiod length had significant beneficial influence on feed efficiency, crop size, pH and *Lactobacillus* spp. abundance which could have associated potential health benefits in broiler chickens.

The third objective to evaluate effect of different feed ingredients offered at *ad-lib* or three times per day meal feeding when diet was finely or coarsely grounded on crop and ileal microbiota was also accomplished here. Meal feeding significantly increased relative crop size and an array of *Lactobacillus* species versus *ad-lib* fed broiler birds. Also, qPCR, specified diet as primary factor influencing crop microbiota. A number of interactions among screen size, feeding behavior and diet composition were noticed, making specific conclusions difficult, however, it is clear these factors play a crucial role in broiler chicken upper gut microbiome.

Implications of present work for broiler management industry

Although work conducted here, alone does not indicate that reducing photoperiod improved immune function and bird health, when combined with a significant body of evidence reported previously on this topic does further support this concept. Furthermore, our subsequent studies have advanced knowledge in this field by extending the notion that photoperiod and health are linked potentially beyond the hormonal regulation of immune cells via melatonin, to include the upper intestinal tract microbiome. Based on the present work positive effects on infectious mortalities and abundance of *Lactobacillus* spp. in the crop was observed with relatively short photoperiods (13L) which might not be a commercially feasible, considering the modern broiler chicken performance targets. However, similar to photoperiod, during this work several other major management factors including feed ingredient selection, processing and amino acid content demonstrated significant role in crop microbial ecology alterations. Thus in combination with less restricted photoperiod adjustment, these strategies could be employed commercially to promote upper gut health and bird livability. Moreover, these strategies may be critical for poultry farmers, in today's era of reduced availability of antibiotics except for treatment of confirmed bacterial infection.

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